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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
G01N 33/50, 33/68, C12N 9/12

A1

(11) International Publication Number: **WO 98/30902**

(43) International Publication Date: 16 July 1998 (16.07.98)

(21) International Application Number: PCT/GB98/00095

(22) International Filing Date: 13 January 1998 (13.01.98)

(30) Priority Data:

| | | |
|---|---|---|
| 9700574.8 | 13 January 1997 (13.01.97) | GB |
| 9713131.2 | 20 June 1997 (20.06.97) | GB |

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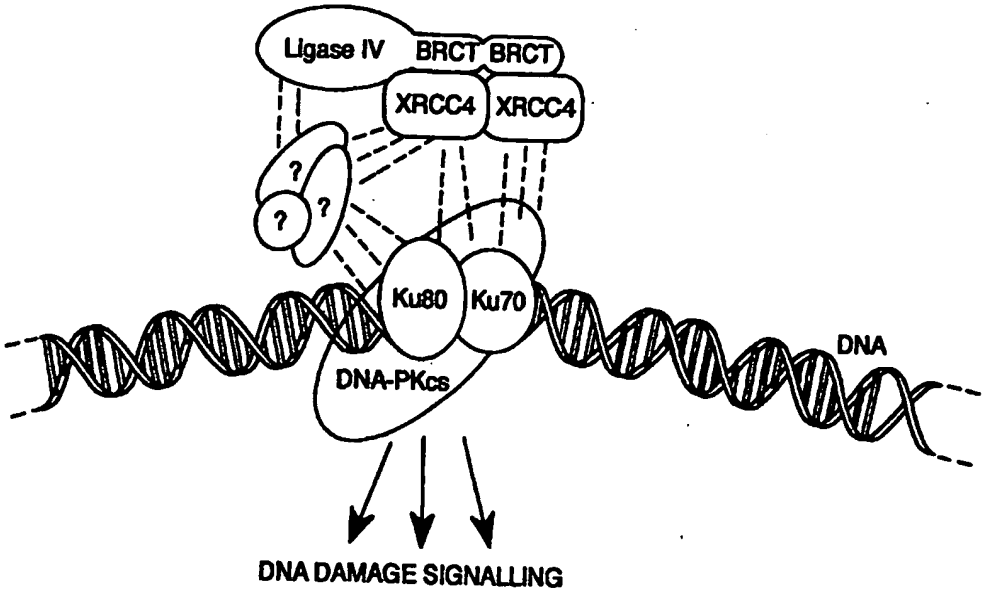
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: ASSAYS, AGENTS, THERAPY AND DIAGNOSIS RELATING TO MODULATION OF CELLULAR DNA REPAIR ACTIVITY




(57) Abstract

A yeast homologue of mammalian DNA ligase IV is provided and a role for DNA ligase IV established in the Ku–associated DNA repair pathway. Additionally interactions between DNA ligase IV and XRCC4, and interaction between XRCC4 and DNA–PKcs/Ku are established, providing for assays for agents which modulate such interactions and therefore cellular DNA repair activity. Such agents are useful in treatment of cancers, retroviral infections, immune system disorders and other conditions in which cellular DNA repair activity plays a role. Individuals with a predisposition to a disorder in which DNA repair plays a role may be diagnosed, by screening for the presence or absence of a defect in XRCC4 and/or DNA ligase IV activity.

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# ASSAYS, AGENTS, THERAPY AND DIAGNOSIS RELATING TO MODULATION OF CELLULAR DNA REPAIR ACTIVITY

The present invention relates to screening methods, peptides, mimetics, and methods of use based on the surprising discovery and characterisation of an interaction between known proteins and the establishment that such interaction plays a key role in DNA repair, and thus numerous cellular processes of interest in therapeutic contexts. Two proteins in question are XRCC4 and DNA ligase IV. Interaction between XRCC4 and DNA-PKcs/Ku is also indicated.

The invention has arisen on the basis of the work of the present inventors establishing for the first time crucial information about XRCC4. Some information was available on the physiological function of this protein, it having been implicated in the Ku-associated DNA double-strand break repair (KADR) apparatus. However, very little was known about its biological activity and what its role in the KADR apparatus actually is. Prior to the making of the present invention it was not feasible to provide assays useful as primary screens for inhibitors of XRCC4.

Furthermore, the inventors' new cloning work has identified a yeast homologue of mammalian DNA ligase IV. No physiological function has previously been assigned to mammalian DNA ligase IV, but the inventors' yeast work, including analysis of the effect of knock-out mutation in yeast, now establishes the physiological relevance of DNA ligase IV and thus provides indication of therapeutic contexts in which modulation of its function can be effected.

The work disclosed herein establishing interaction between XRCC4 and DNA ligase IV, interaction between XRCC4 and DNA-PKcs/Ku, and also a biological role for such interactions, now gives rise to screening methods for identifying compounds which affect the interaction,

particularly those which interfere with it, and which may affect or modulate particular aspects of cellular DNA repair activity, useful in a therapeutic context, for example in the treatment of proliferative disorders, cancers and tumours, disorders involving retroviruses such as AIDS, human adult T-cell leukaemia/lymphoma, Type I diabetes and multiple sclerosis, and also in radiotherapy. Furthermore it gives rise to the rational design of peptides or mimetics or functional analogues which fulfil this function.

One of the most dangerous forms of damage that can befall a cell is the DNA double-strand break (DSB), which is the principal lethal lesion induced by ionising radiation and by radiomimetic agents. Consequently, cells have evolved highly effective and complex systems for recognising this type of DNA damage and ensuring that it is repaired efficiently and accurately. Two major pathways have evolved to repair DNA DSBs in eukaryotes, homologous recombination and DNA non-homologous end-joining (NHEJ).

Much of what is currently known about DNA NHEJ in mammalian systems has been obtained through studies of a series of mutant rodent cell lines that were identified originally as being hypersensitive towards ionising radiation and which display severe defects in DNA DSB repair (reviewed in Jeggo *et al.*, 1995; Roth *et al.*, 1995). Characterisation of these cell lines has revealed that they fall into three complementation groups, termed IR4, IR5 and IR7. The hamster cell line XR-1 defines IR4, IR5 consists of a number of independently isolated hamster cell mutants, and IR7 contains the hamster cell line V3 and cells derived from the severe combined immune-deficient (scid) mouse. Various studies have shown that IR4, IR5 and IR5 cells are defective in antibody and T-cell receptor V(D)J recombination.

Considerable effort has been directed towards

establishing the nature of the gene-products defective in cells of IR4, IR5 and IR7, and determining how they function in DNA NHEJ. As a result of such studies, it was shown that cells of IR5 and IR7 are deficient in components of the DNA-dependent protein kinase (DNA-PK) (Ku80 and DNA-PKcs, respectively). DNA-PK is a nuclear protein Ser/Thr kinase that displays the unusual property of being activated upon binding to DNA DSBs or other perturbations of the DNA double-helix (Jackson, 1997). In light of the biochemical properties of DNA-PK which have been established, an attractive hypothesis is that this enzyme serves as a DNA damage sensor *in vivo*.

In contrast to cells of IR5 and IR7, XR-1 cells of IR4 are not deficient in a DNA-PK component, as evidenced by the fact that extracts of these cells have normal DNA end-binding activity (Getts and Stamato, 1994; Rathmell and Chu, 1994; Finnie *et al.*, 1996) and DNA-PK activity (Blunt *et al.*, 1995), and that expression of neither Ku80 nor DNA-PKcs complements the V(D)J recombination or radiosensitivity defects of XR-1 cells (Taccioli *et al.*, 1994; Blunt *et al.*, 1995). Instead, it has been shown that DNA from human chromosome region 5q13-14 complements XR-1 cells, the complementing gene being termed *XRCC4* (Otevrel and Stamato, 1995).

Furthermore, (Li *et al.*, 1995) have identified the *XRCC4* gene recently through its ability to confer normal V(D)J recombination activity and partially restore the DSB repair defect on XR-1 cells, and have demonstrated that the *XRCC4* locus is deleted in XR-1 cells.

Interestingly, *XRCC4* encodes a small 334 amino acid residue protein of calculated molecular weight of 38 kDa, and the human and mouse homologues of this protein have been shown to be approximately 75% identical (Li *et al.*, 1995). Perhaps surprisingly, however, sequence analyses reveal that XRCC4 is not significantly related to any previously-

characterised proteins. Therefore, although it is clear that XRCC4 plays a crucial role in DNA DSB repair and V(D)J recombination, the cloning and sequencing of the cDNA for this factor has so far provided little clue to its mechanism of action.

The Li *et al.* paper is the only paper published on the XRCC4 protein as such prior to the priority date of the present invention. It reports that XRCC4 is not related to any other proteins and so its sequence gives no clear clues as to its function. Prior to the present work, therefore, the only assays available for XRCC4 were cellular radiosensitivity and cellular V(D)J recombination - assays that cannot be used as primary screens for inhibitors. Consequently, it was impossible to conceive of any biochemical screen for the activity of this factor.

It should be noted too that the Li *et al.* paper does not provide any evidence that XRCC4 is a nuclear protein (shown herein) and discusses on page 1084 that XRCC4 has putative sites for cytoplasmic protein tyrosine kinases. Thus, it is clear that there really was nothing known about how this protein might act.

The present inventors have shown that XRCC4 exists, at least in part, in the cell nucleus and demonstrated convincingly that it interacts with DNA ligase IV, and also DNA-PKcs/Ku. Evidence is provided herein in the experimental section, with confirmation being provided also by Mizuta *et al.*, 1997. Grawunder *et al*, 1997 has also provided evidence of interaction between XRCC4 and DNA ligase IV. See also the inventors' publications Teo and Jackson, 1997 and Critchlow *et al.* 1997.

DNA ligases are catalysts which join together Okazaki fragments during lagging strand DNA synthesis, complete exchange events between homologous duplex DNA molecules, and seal single- or double-strand breaks in the DNA that are

produced either by the direct action of a DNA damaging agents or by DNA repair enzymes removing DNA lesions (for review, see Lindahl and Barnes, 1992). In contrast to prokaryotic and yeast systems, where only a single species of DNA ligase has been previously been described (Johnston and Nasmyth, 1978), four biochemically distinct DNA ligases have been identified in mammalian cells (Tomkinson *et al.*, 1991; Wei *et al.*, 1995; Robins and Lindahl, 1996). *In vitro* assays, and studies of yeast and human cells containing mutated alleles of DNA ligase I suggest that this enzyme joins Okazaki fragments during DNA replication (Henderson *et al.*, 1985; Malkas *et al.*, 1990; Tomkinson *et al.*, 1991; Barnes *et al.*, 1992; Li *et al.*, 1994; Prigent *et al.*, 1994; Waga *et al.*, 1994). Furthermore, the sensitivity of DNA ligase I mutant cells to ultraviolet (UV) irradiation and some DNA damaging agents suggests that DNA ligase I is involved in nucleotide excision repair and base excision repair (Henderson *et al.*, 1985; Lehmann *et al.*, 1988; Malkas *et al.*, 1990; Tomkinson *et al.*, 1991; Barnes *et al.*, 1992; Li *et al.*, 1994; Prigent *et al.*, 1994; Waga *et al.*, 1994).

Much less, however, is known about the function of the other three mammalian DNA ligases. It is currently unclear whether DNA ligase II and III arise from separate genes or by alternative splicing of the same gene (Roberts *et al.*, 1994; Wang *et al.*, 1994; Husain *et al.*, 1995). However, ligase II is induced in response to alkylation damage (Creissen and Shall, 1982), suggesting a role in DNA repair. Similarly, the elevation of a splice variant of ligase III (ligase III-β) levels in spermatocytes undergoing meiotic recombination (Chen *et al.*, 1995; Husain *et al.*, 1995; Mackey *et al.*, 1997) and the association of another splice variant (ligase III-α) with the DNA repair protein XRCC1 (Caldecott *et al.*, 1994; Thompson *et al.*, 1990) are consistent with this enzyme joining DNA strand breaks to

complete DNA recombination and repair (Jessberger *et al.*, 1993). Indeed, DNA ligase III, when present in a complex with XRCC-1, can reconstitute the ligation event necessary to complete base excision repair *in vitro* (Kubota *et al.*, 1996).

A fourth enzyme, DNA ligase IV, has been purified recently from human cells and has distinct biochemical properties from other ligases (Robins and Lindahl, 1996). The physiological function of mammalian ligase IV is, however, unknown.

In most prokaryotes there is only one DNA ligase, and this enzyme catalyses all the DNA-joining events during replication, recombination and repair (Lindahl and Barnes, 1992). Similarly, genetic and biochemical data have suggested that there is only one DNA ligase in *Saccharomyces cerevisiae* (Lindahl and Barnes, 1992), although fractionation of yeast cell extracts has given an indication of a second DNA ligase activity (Tomkinson *et al.*, 1992).

The present inventors searched for DNA ligase homologues in the *S. cerevisiae* genome, which was completely sequenced recently (Goffeau *et al.*, 1996; Oliver, 1996). These searches identified a hitherto uncharacterised open reading frame (ORF) with sequence similarity along its entire length to mammalian DNA ligase IV. The experimental section below describes the effects of disrupting this gene, which the inventors have termed *LIG4*, on DNA replication, homologous recombination, and DNA repair in response to a variety of DNA-damaging agents. These studies show that *LIG4* plays a crucial role in DNA double-strand break repair via the non-homologous end-joining (NHEJ) pathway but does not have an essential role in other DNA repair pathways studied.

Furthermore, it is shown that *LIG4* functions in the same DNA repair pathway that utilises the DNA end-binding protein Ku. However, the phenotype of *lig4* mutant yeasts is

not identical to those of yeasts disrupted for Ku function, revealing that Ku has additional roles in genome maintenance.

In summary, XRCC4 was known to be involved somehow in Ku-associated DNA double-strand break repair (KADR), but its biological activity was obscure. The present inventors have established for the first time biological activity of XRCC4, that is binding to DNA ligase IV. Furthermore, the physiological relevance of DNA ligase IV was not known. The inventors have now established that DNA ligase IV is important for double-strand DNA break repair via non-homologous end joining (NHEJ) - by unexpectedly identifying and cloning, then mutating, a yeast homologue gene and by establishing strong interaction between XRCC4 and DNA ligase IV.

The inventors have also established that XRCC4 interacts with DNA-PKcs/Ku, and shown that DNA-PKcs is able to phosphorylate XRCC4.

Based on this and other work described below, the present invention in various aspects provides for modulation of interaction between XRCC4 and DNA ligase IV.

Various aspect the present invention provide for the use of XRCC4 and DNA ligase IV in screening methods and assays for agents which modulate interaction between XRCC4 and DNA ligase IV.

Further aspects provide for modulation of interaction between XRCC4 and DNA-PKcs/Ku and use of these molecules in screening methods and assays for agents which modulate interaction between XRCC4 and DNA-PKcs/Ku. For simplicity, much of the present disclosure refers to XRCC4 and DNA ligase IV. However, unless the context requires otherwise, every such reference should be taken to be equally applicable to the interaction between XRCC4 and DNA-PKcs/Ku.

Methods of obtaining agents able to modulate interaction between XRCC4 and DNA ligase IV (or, it must be remembered, XRCC4 and DNA-PKcs/Ku) include methods wherein a suitable end-point is used to assess interaction in the presence and absence of a test substance. Detailed disclosure in this respect is included below. It is worth noting, however, that combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate bind to and/or activity of a polypeptide. Such libraries and their use are known in the art, for all manner of natural products, small molecules and peptides, among others. The use of peptide libraries may be preferred in certain circumstances.

Appropriate agents may be obtained, designed and used for any of a variety of purposes.

One is anti-tumour or anti-cancer therapy, particularly augmentation of radiotherapy or chemotherapy. Ionising radiation and radiomimetic drugs are commonly used to treat cancer by inflicting DNA damage. Cells deficient in DNA repair, particularly the KADR pathway, are hypersensitive to ionising radiation and radiomimetics. Evidence provided herein shows the KADR pathway involves XRCC4 and DNA ligase IV, indicating that inhibition of their function, e.g. by inhibiting their interaction, will have an effect on the KADR pathway, DNA repair and cellular sensitivity to ionising radiation and radiomimetics.

Another is the potentiation of gene targeting and gene therapy. Inhibition of KADR may be used to increase efficiencies of gene targeting, of interest and ultimate use in gene therapy. Two ways exist for repairing DNA double-stranded breaks (DSBs). One is through the process of illegitimate recombination (also known as DNA non-homologous end-joining or NHEJ) and this is catalysed by the KADR

system now known to involve XRCC4 and DNA ligase IV. The other system is the process of homologous recombination, whereby the damaged DNA molecule exchanges information with an undamaged homologous partner DNA molecule. In mammalian cells, the illegitimate pathway tends to predominate. Inhibiting the KADR system will make the proportion of DSBs repaired by homologous recombination increase. Thus, anti-KADR factor agents, including those provided in accordance with the present invention, will have this effect.

Homologous gene targeting is used in making knock-out mice and other transgenic animals but it is not very efficient, so increasing this efficiency in accordance with the present invention will be highly beneficial. Ultimately, gene therapist wish to precisely replace the mutated gene with a functional one. At present just to get the functional gene to integrate anywhere in the genome is the priority, but the long-term aim is for integration at the right site. KADR (e.g. XRCC4 and/or ligase IV, or XRCC4 and/or DNA-PKcs/Ku) inhibitors therefore have a great therapeutic potential in such context.

A further, related, purpose is in anti-retroviral therapy, since DNA repair pathways such as involving KADR and the components XRCC4 and DNA ligase IV are involved in effecting retroviral and retrotransposon integration into the genome of a host cell. Retroviruses are of considerable risk to the health of humans and animals, causing, *inter alia*, AIDS, various cancers and human adult T-cell leukaemia/lymphoma. Integration of retroviral DNA into the genome is essential for efficient viral propagation and may be targeted by inhibition of DNA repair pathway components.

Additionally, modulators of KADR components such as XRCC4 and DNA ligase IV, DNA-PKcs/Ku, may be used in modulation of immune system function, since such factors are required for generation of mature immunoglobulin and T-cell receptor genes by site-specific V(D)J recombination.

Compounds which stabilise the interaction between two components, such as XRCC4 and DNA ligase IV, or XRCC4 and DNA-PKcs/Ku, and which may up-regulate activity, may be screened for using assays in which conditions are too harsh for the relevant interaction. Agents which stabilise the interaction may be identified. One alternative is to screen for substances that enhance DNA ligase IV catalytic activity, which may be determined as discussed elsewhere. An up-regulator of activity may be used to potentiate DNA repair further, and this may be in normal individuals, with possible long-term beneficial effects bearing in mind that many of the common manifestations of ageing arise through the gradual and inexorable accumulation of mutations in somatic cells. Up-regulators may be used in treating patients who are debilited in the KADR pathway or other DNA repair pathway.

Interaction between XRCC4 and DNA ligase IV, or XRCC4 and DNA-Pkcs/Ku may be inhibited by inhibition of the production of the relevant protein. For instance, production of one or more of these components may be inhibited by using appropriate nucleic acid to influence expression by antisense regulation. The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established. Double-stranded DNA is placed under the control of a promoter in a "reverse orientation" such that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works.

Another possibility is that nucleic acid is used which on transcription produces a ribozyme, able to cut nucleic acid at a specific site - thus also useful in influencing gene expression. Background references for ribozymes include Kashani-Sabet and Scanlon, 1995, *Cancer Gene Therapy*, **2**(3): 213-223, and Mercola and Cohen, 1995, *Cancer Gene Therapy*, **2**(1), 47-59.

Thus, various methods and uses of modulators, particularly inhibitors, of XRCC4 and DNA ligase IV, or XRCC4 and DNA-PKcs/Ku, interaction and/or activity are provided as further aspects of the present invention. The purpose of disruption, interference with or modulation of interaction between XRCC4 and DNA ligase IV, and/or XRCC4 and DNA-PKcs/Ku, may be to modulate any activity mediated by virtue of such interaction, as discussed above and further below.

*Brief Description of the Figures*

Figure 1 illustrates co-purification of XRCC4 and DNA ligase IV from HeLa cells.

Figure 1A shows the results of quantitative Western immunoblot analyses for DNA ligase IV (diamonds) and XRCC4 (squares) (percent of protein) for various fractions at each chromatographic stage of gel chromatography filtration for DNA ligase IV purification from HeLa cells.

Figure 1B shows results of quantitative Western immunoblot analyses for DNA ligase IV (diamonds), XRCC4 (squares) and DNA ligase III (circles) (percent of protein) for various fractions at each stage of Mono S column fractionation on the fraction marked with an asterisk in Figure 1A.

Figure 2 shows that YOR005c encodes a homologue of mammalian DNA ligase IV, indicating amino acid sequence similarities between *S. cerevisiae* Lig4p (scLIG4; the

product of the YOR005c ORF) and human DNA ligase IV (hLIGIV). The alignment was generated using the PILEUP programme on the GCG (Genetics Computer Group, Wisconsin) package, and identical and similar amino acid residues are indicated by reverse shading and grey shading, respectively, using the BOXSHADE programme. Amino acid residues are numbered from the amino termini of the full-length polypeptides. Gaps were introduced for maximum alignment. The active site lysine residue is indicated with an arrowhead. The "core" conserved region of DNA ligases of eukaryotes and eukaryotic viruses is delineated with a bar.

Figure 3 shows that *LIG4* functions in the Ku-dependent pathway for repairing ionising radiation-induced DNA damage. The sensitivity of various yeast strains to killing by ionising radiation was judged by exposure to various radiation doses. Error bars are not shown for simplicity; standard deviation is < 5 % of each value point.

Figure 3A shows % cell survival for wild-type, *lig4* mutants and *rad52* mutants at various doses in kRads of ionising radiation.

Figure 3B shows % cell survival for *lig4/yku70* mutants, *rad52* mutants rad52*/lig4* mutants, *rad52/yku70* mutants and *rad52/lig4/yku70* mutants at various doses in kRads of ionising radiation.

Figure 4 shows that disruption of *LIG4* results in a dramatic reduction in the ability to repair restriction enzyme generated cohesive DNA DSBs in plasmid (pBTM116) DNA, plotting transformant recovery relative to uncut control plasmid where various yeast strains, wild-type and mutant, were transformed with pBTM116 digested with *Eco*RI (left panel) or *Pst*I (right panel).

Figure 5 shows a model in which XRCC4 serves as a molecular bridge to target DNA ligase IV to a DNA DSB.

Figure 6 shows the amino acid sequence and encoding nucleotide sequence for *S. cerevisiae LIG4*, provided in

accordance with aspects of the present invention. Translation begins at the start site indicated by the arrow.

Amino acid and nucleic acid sequences of polypeptides useful in various aspects of the present invention are available from GenBank under the following accession numbers: human Ku70 - J04611; human Ku80 - M30938; *S. cerevisiae* Ku70 - X70379; *S. cerevisiae* Ku80 - Z49702; human ligase IV - X83441; *S. cerevisiae* ligase IV - YOR005c on the right arm of *S. cerevisiae* chromosome XV, accession number Z74913; human XRCC4 - U40622 (334 amino acid residue open reading frame); human DNA-Pkcs - U47077 (Hartley *et al.* originally provided the sequence, though lacking an intron. Poltoratsky *et al.* provided a partial sequence including the intron not included in the Hartley *et al.* sequence. The sequence available from GenBank is complete.).

All documents and GenBank sequences mentioned anywhere in this specification are incorporated by reference.

The present invention in various aspects provides for modulating, interfering with or interrupting interacion between the XRCC4 protein and DNA ligase IV, using an appropriate agent. The present invention also provides in analogous aspects for modulating, interfering with or interrupting interaction between the XRCC4 protein and DNA-Pkcs/Ku, using an appropriate agent.

Such an agent capable of modulating interaction between XRCC4 and DNA ligase IV may be capable of blocking binding between a site located within amino acid residues 550-884 of human DNA ligase IV, which may be at one or other or both of the BRCT domains (discussed further below), or between these domains, and a site on human XRCC4. The site on DNA ligase IV may be between amino acid residues 591-676, between amino

acid residues 728-844 or between residues 677-727. The full amino acid sequence of the XRCC4 protein has been elucidated and is set out in Li *et al.* (*Cell* (1995) **83**, 1079-1089) which is incorporated herein by reference, and of which the amino acid residue numbering is used along with the encoding nucleic acid sequence. The GenBank reference is indicated above. The DNA ligase IV amino acid and nucleotide coding sequences are given in Wei *et al.*, of which the amino acid residue numbering is used, with the yeast *LIG4* sequences being shown in Figure 6. The GenBank references are given above.

Note, that recently Wilson, T.E. 1997, *Nature* **388**: 495-498 has suggested that the initiating methionine for human DNA ligase IV is upstream from that indicated by Wei *et al.*, 1995, whose amino acid sequence numbering is used herein. See the Wilson paper for details. The present inventors have preliminary data which disagrees with that of Wilson. However, should Wilson turn out to be correct this would have no bearing on any aspect of the present invention. The presence or absence of additional amino acids at the N-terminus of DNA ligase IV is unlikely to have any effect on its interaction with XRCC4, and the fact remains that the present inventors' work shows interaction of XRCC4 with the DNA ligase IV which occurs in human cells.

Agents may be identified by screening techniques which involve determining whether an agent under test inhibits or disrupts the binding of DNA ligase IV protein or a suitable fragment thereof (e.g. including amino acid residues 550-884, residues 591-676, residues 728-844 or residues 677-727, or a smaller fragment of any of these regions) of human DNA ligase IV, with XRCC4 or a fragment thereof, or a suitable analogue, fragment or variant thereof.

Suitable fragments of XRCC4 or DNA ligase IV include

those which include residues which interact with the counterpart protein. Smaller fragments, and analogues and variants of this fragment may similarly be employed, e.g. as identified using techniques such as deletion analysis or alanine scanning.

Thus, the present invention provides a peptide fragment of XRCC4 which is able to bind DNA ligase IV and/or inhibit interaction between XRCC4 and DNA ligase IV, and provides a peptide fragment of DNA ligase IV which is able to bind DNA ligase IV and/or inhibit interaction between DNA ligase IV and XRCC4, such peptide fragments being obtainable by means of deletion analysis and/or alanine scanning of the relevant protein - making an appropriate mutation in sequence, bringing together a mutated fragment of one of the proteins with the other or a fragment thereof and determining interaction. In preferred embodiments, the peptide is short, as discussed below, and may be a minimal portion that is able to interact with the relevant counterpart protein and/or inhibit the relevant interaction.

Of course, similar considerations apply to XRRC4 and DNA-PKcs/Ku interacting portions.

Screening methods and assays are discussed in further detail below.

One class of agents that can be used to disrupt the binding of XRCC4 and DNA ligase IV are peptides based on the sequence motifs of XRCC4 or DNA ligase IV that interact with counterpart DNA ligase IV or XRCC4. Such peptides tend to be short, and may be about 40 amino acids in length or less, preferably about 35 amino acids in length or less, more preferably about 30 amino acids in length, or less, more preferably about 25 amino acids or less, more preferably about 20 amino acids or less, more preferably about 15 amino acids or less, more preferably about 10 amino acids or less,

or 9, 8, 7, 6, 5 or less in length. The present invention also encompasses peptides which are sequence variants or derivatives of a wild type XRCC4 or DNA ligase IV sequence, but which retain ability to interact with DNA ligase IV or XRCC4 (respectively, as the case may be) and/or ability to modulate interaction between XRCC4 and DNA ligase IV.

Instead of using a wild-type XRCC4 or DNA ligase IV fragment, a peptide or polypeptide may include an amino acid sequence which differs by one or more amino acid residues from the wild-type amino acid sequence, by one or more of addition, insertion, deletion and substitution of one or more amino acids. Thus, variants, derivatives, alleles, mutants and homologues, e.g. from other organisms, are included.

Preferably, the amino acid sequence shares homology with a fragment of the relevant XRCC4 or DNA ligase IV fragment sequence shown preferably at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85% homology, or at least about 90% or 95% homology. Thus, a peptide fragment of XRCC4 or DNA ligase IV may include 1, 2, 3, 4, 5, greater than 5, or greater than 10 amino acid alterations such as substitutions with respect to the wild-type sequence.

A derivative of a peptide for which the specific sequence is disclosed herein may be in certain embodiments the same length or shorter than the specific peptide. In other embodiments the peptide sequence or a variant thereof may be included in a larger peptide, as discussed above, which may or may not include an additional portion of XRCC4 or DNA ligase IV. 1, 2, 3, 4 or 5 or more additional amino acids, adjacent to the relevant specific peptide fragment in XRCC4 or DNA ligase IV, or heterologous thereto may be included at one end or both ends of the peptide.

(It should not be forgotten that references to XRCC4 and DNA ligase IV apply equally to XRCC4 and DNA-Pkcs/Ku.)

As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Similarity may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* **215**: 403-10, which is in standard use in the art. Homology may be over the full-length of the relevant peptide or over a contiguous sequence of about 5, 10, 15, 20, 25, 30, 35, 50, 75, 100 or more amino acids, compared with the relevant wild-type amino acid sequence.

As noted, variant peptide sequences and peptide and non-peptide analogues and mimetics may be employed, as discussed further below.

Various aspects of the present invention provide a substance, which may be a single molecule or a composition including two or more components, which includes a peptide fragment of XRCC4 or DNA ligase IV which includes a sequence as included in the relevant GenBank entry, a peptide consisting essentially of such a sequence, a peptide including a variant, derivative or analogue sequence, or a non-peptide analogue or mimetic which has the ability to bind XRCC4 or DNA ligase IV and/or modulate, disrupt or interfere with interaction between XRCC4 or DNA ligase IV.

Variants include peptides in which individual amino acids can be substituted by other amino acids which are closely related as is understood in the art and indicated above.

Non-peptide mimetics of peptides are discussed further below.

As noted, a peptide according to the present invention

and for use in various aspects of the present invention may include or consist essentially of a fragment of XRCC4 or DNA ligase IV as disclosed, such as a fragment whose sequence is included in the relevant GenBank entry. Where one or more additional amino acids are included, such amino acids may be from XRCC4 or DNA ligase IV or may be heterologous or foreign to XRCC4 or DNA ligase IV. A peptide may also be included within a larger fusion protein, particularly where the peptide is fused to a non-XRCC4 or DNA ligase IV (i.e. heterologous or foreign) sequence, such as a polypeptide or protein domain.

The invention also includes derivatives of the peptides, including the peptide linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule, and/or a targeting molecule such as an antibody or binding fragment thereof or other ligand. Techniques for coupling the peptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art. In one embodiment, the carrier molecule is a 16 aa peptide sequence derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin"), which can be coupled to a peptide via a terminal Cys residue. The "Penetratin" molecule and its properties are described in WO 91/18981.

Peptides may be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be

prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Another convenient way of producing a peptidyl molecule according to the present invention (peptide or polypeptide) is to express nucleic acid encoding it, by use of nucleic acid in an expression system.

Accordingly the present invention also provides in various aspects nucleic acid encoding the polypeptides and peptides of the invention.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

Nucleic acid sequences encoding a polypeptide or peptide in accordance with the present invention can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992), given the nucleic acid sequence and clones available. These

techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding XRCC4 or DNA ligase IV fragments may be generated and used in any suitable way known to those of skill in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Modifications to the XRCC4 or DNA ligase IV sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified XRCC4 or DNA ligase IV peptide or to take account of codon preference in the host cells used to express the nucleic acid.

In order to obtain expression of the nucleic acid sequences, the sequences can be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide produced in the host cell is secreted from the cell. Polypeptide can then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the polypeptide is produced and recovering the polypeptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of E. coli, yeast, and eukaryotic cells

such as COS or CHO cells.

Thus, the present invention also encompasses a method of making a polypeptide or peptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide or peptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides and peptides may also be expressed in in vitro systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is E. coli.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing heterologous nucleic acid as

disclosed herein.

The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide (or peptide) is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a

polypeptide or peptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid encoding a peptidyl molecule according to the present invention may take place in vivo by way of gene therapy, to disrupt or interfere with interaction between XRCC4 or DNA ligase IV

Thus, a host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place in vivo or ex vivo), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or birds comprising such a cell are also provided as further aspects of the present invention.

This may have a therapeutic aim. (Gene therapy is discussed below.) Also, the presence of a mutant, allele, derivative or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying substances which modulate activity of the encoded polypeptide *in vitro* or are otherwise indicated to be of therapeutic potential. Knock-out mice, for instance, may be used to test for radiosensitivity. Conveniently, however, at least preliminary assays for such

substances may be carried out *in vitro*, that is within host cells or in cell-free systems. Where an effect of a test compound is established on cells*in vitro*, those cells or cells of the same or similar type may be grafted into an appropriate host animal for *in vivo* testing.

Suitable screening methods are conventional in the art. They include techniques such as radioimmunosassay, scintillation proximetry assay and ELISA methods. Suitably either the XRCC4 protein or fragment or DNA ligase IV or fragment, or an analogue, derivative, variant or functional mimetic thereof, is immobilised whereupon the other is applied in the presence of the agents under test. In a scintillation proximetry assay a biotinylated protein fragment is bound to streptavidin coated scintillant - impregnated beads (produced by Amersham). Binding of radiolabelled peptide is then measured by determination of radioactivity induced scintillation as the radioactive peptide binds to the immobilized fragment. Agents which intercept this are thus inhibitors of the interaction. Further ways and means of screening for agents which modulate interaction between XRCC4 and DNA ligase IV are discussed below.

In one general aspect, the present invention provides an assay method for a substance with ability to modulate, e.g. disrupt or interfere with interaction or binding between XRCC4 and DNA ligase IV, the method including:

(a) bringing into contact a substance according to the invention including a peptide fragment of XRCC4 or a derivative, variant or analogue thereof as disclosed, a substance including the relevant fragment of DNA ligase IV or a variant, derivative or analogue thereof, and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of interaction or binding

of said substances, said substances interact or bind; and

(b) determining interaction or binding between said substances.

A test compound which disrupts, reduces, interferes with or wholly or partially abolishes binding or interaction between said substances (e.g. including a XRCC4 fragment and including a DNA ligase IV fragment), and which may modulate XRCC4 and/or DNA ligase IV activity, may thus be identified.

Another general aspect of the present invention provides an assay method for a substance able to bind the relevant region of XRCC4 or DNA ligase IV as the case may be, the method including:

(a) bringing into contact a substance which includes a peptide fragment of XRCC4 which interacts with DNA ligase IV as disclosed, or which includes a peptide fragment of DNA ligase IV which interacts with XRCC4, or a variant, derivative or analogue of such peptide fragment, as disclosed, and a test compound; and

(b) determining binding between said substance and the test compound.

A test compound found to bind to the relevant portion of XRCC4 may be tested for ability to modulate, e.g. disrupt or interfere with, XRCC4 interaction or binding with DNA ligase IV and/or ability to affect DNA ligase IV and/or XRCC4 activity or other activity mediated by XRCC4 or DNA ligase IV as discussed already above.

Similarly, a test compound found to bind the relevant portion of DNA ligase IV may be tested for abiliy to modulate, e.g. disrupt or interfere with, DNA ligase IV interaction or binding with XRCC4 and/or ability to affect XRCC4 and/or DNA ligase IV activity or other activity mediated by DNA ligase IV or XRCC4 as discussed already above.

These aspects apply equally to interaction between DNA-

Pkcs/Ku and XRCC4. Furthermore, since DNA-PKcs phosphorylates XRCC4, determining of such phosphorylation can be used in an appropriate assay.

A further aspect of the present invention provides an assay method including

(a) bringing into contact a substance which includes at least a fragment of DNA-PKcs/Ku which phosphorylates XRCC4, a substance which includes at least a fragment of XRCC4 including a site phosphorylated by DNA-PKcs/Ku, and a test compound; and

(b) determining phosphorylation at said site.

Of course, any suitable variant or derivative of DNA-PKcs/Ku and/or XRCC4 may be employed in such an assay.

Phosphorylation may be determined for example by immobilising XRCC4 or a fragment, variant or derivative thereof, e.g. on a bead or plate, and detecting phosphorylation using an antibody or other binding molecule which binds the relevant site of phosphorylation with a different affinity when the site is phosphorylated from when the site is not phosphorylated. Such antibodies may be obtained by means of any standard technique as discussed elsewhere herein, e.g. using a phosphorylated peptide (such as a fragment of XRCC4). Binding of a binding molecule which discriminates between the phosphorylated and non-phosphorylated form of XRCC4 or relevant fragment, variant or derivative thereof may be assessed using any technique available to those skilled in the art, which may involve determination of the presence of a suitable label, such as fluorescence. Phosphorylation may be determined by immobilisation of XRCC4 or a fragment, variant or derivative thereof, on a suitable substrate such as a bead or plate, wherein the substrate is impregnated with scintillant, such as in a standard scintillation proximetry assay, with phosphorylation being determined via measurement of the incorporation of radioactive phosphate. Rather than immobilising XRCC4, its phosphorylation by DNA-PKcs/Ku may

be assayed by means of allowing its radio- or other labelling in solution, with a suitable specific binding member such as an antibody or DNA ligase IV or an XRCC4-binding fragment thereof being used to pull it out for determination of labelling. Phosphate incorporation into XRCC4 or a fragment, variant or derivative thereof, may be determined by precipitation with acid, such as trichloroacetic acid, and collection of the precipitate on a suitable material such as nitrocellulose filter paper, followed by measurement of incorporation of radiolabeled phosphate. SDS-PAGE separation of substrate may be employed followed by detection of radiolabel.

Another general aspect of the present invention provides an assay method for a substance able to affect DNA ligase IV activity, the method including:

(a) bringing into contact DNA ligase IV and a test compound; and

(b) determining DNA ligase IV activity.

DNA ligase IV activity may be determined in the presence and absence of XRCC4 to allow for an effect of a test compound on activity to be attributed to an effect on interaction between DNA ligase IV and XRCC4.

DNA ligase IV activity may be conveniently determined by means of its adenylation. DNA ligase IV may be incubated with radiolabelled ATP (e.g. as described below) or any suitable ATP analogue that interacts with DNA ligase IV in an analogous manner, so that radiolabel is incorporated into the ligase. (The ligase goes through an enzyme-AMP adenylated intermediate.) Such radiolabel incorporation may be detected by various approaches, including for example scintillation proximetry assay Thus, radiolabelling of DNA ligase IV may be determined in the presence and absence of test compound and in the presence and absence of XRCC4. Pre-adenylation of DNA ligase IV with radiolabel allows for

assaying for discharge of the radiolabel.

Another activity of DNA ligase IV which may be determined is DNA ligase IV-mediated DNA strand joining. For instance, two DNA molecules may be provided each of which includes a site to which a PCR primer anneals under appropriate conditions. When the two DNA molecules are covalently linked by DNA ligase IV to form a single DNA molecule, a PCR template results which can be amplified using the primers. No PCR product results in the absence of ligation. The amount of PCR product obtained in a given reaction can be quantitated with respect to DNA ligase activity. Another option is to attach a DNA molecule to an insoluble support and to add another, labelled DNA molecule. Following addition of DNA ligase IV in the presence or absence of a test compound and a washing step, attachment of the second molecule to the support, which can only take place via ligation to the DNA molecule bound to the support, can be determined by means of the label and related to DNA ligase IV activity. A further assay may include DNA end-joining, e.g. as described by Gawunder *et al.*, 1997.

A substance found to be able to modulate DNA ligase IV activity, e.g. in the presence or absence of XRCC4, may be employed in a similar assay using DNA ligase I and/or DNA ligase III, in order to assess specificity for DNA ligase IV.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive for ability to modulate interaction between XRCC4 and DNA ligase IV and/or inhibit XRCC4 or DNA ligase IV activity or a mediated activity.

The precise format of an assay of the invention may be varied by those of skill in the art using routine skill and

knowledge. For example, interaction between substances may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels, especially for petidyl substances include $^{35}S$-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody.

The protein which is immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

An assay according to the present invention may also take the form of an *in vivo* assay. The *in vivo* assay may be performed in a cell line such as a yeast strain or mammalian cell line in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

The ability of a test compound to modulate interaction or binding between XRCC4 and DNA ligase IV may be determined using a so-called two-hybid assay.

For example, a polypeptide or peptide containing a

fragment of XRCC4 or DNA ligase IV as the case may be, or a peptidyl analogue or variant thereof as disclosed, may be fused to a DNA binding domain such as that of the yeast transcription factor GAL 4. The GAL 4 transcription factor includes two functional domains. These domains are the DNA binding domain (GAL4DBD) and the GAL4 transcriptional activation domain (GAL4TAD). By fusing one polypeptide or peptide to one of those domains and another polypeptide or peptide to the respective counterpart, a functional GAL 4 transcription factor is restored only when two polypeptides or peptides of interest interact. Thus, interaction of the polypeptides or peptides may be measured by the use of a reporter gene probably linked to a GAL 4 DNA binding site which is capable of activating transcription of said reporter gene. This assay format is described by Fields and Song, 1989, Nature 340; 245-246. This type of assay format can be used in both mammalian cells and in yeast. Other combinations of DNA binding domain and transcriptional activation domain are available in the art and may be preferred, such as the LexA DNA binding domain and the VP60 transcriptional activation domain.

To take a Lex/VP60 two hybrid screen by way of example for the purpose of illustration, yeast or mammalian cells may be transformed with a reporter gene construction which expresses a selective marker protein (e.g. encoding β-galactosidase or luciferase). The promoter of that gene is designed such that it contains binding site for the LexA DNA-binding protein. Gene expression from that plasmid is usually very low. Two more expression vectors may be transformed into the yeast containing the selectable marker expression plasmid, one containing the coding sequence for the full length LexA gene linked to a multiple cloning site. This multiple cloning site is used to clone a gene of interest, i.e. encoding a XRCC4 or DNA ligase IV polypeptide or peptide in accordance with the present invention, in

frame on to the LexA coding region. The second expression vector then contains the activation domain of the herpes simplex transactivator VP16 fused to a test peptide sequence or more preferably a library of sequences encoding peptides with diverse e.g. random sequences. Those two plasmids facilitate expression from the reporter construct containing the selectable marker only when the LexA fusion construct interacts with a polypeptide or peptide sequence derived from the peptide library.

A modification of this when looking for peptides or other substances which interfere with interaction between a XRCC4 polypeptide or peptide and DNA ligase IV polypeptide or peptide, employs the XRCC4 or DNA ligase IV polypeptide or peptide as a fusion with the LexA DNA binding domain, and the counterpart DNA ligase IV or XRCC4 polypeptide or peptide as a fusion with VP60, and involves a third expression cassette, which may be on a separate expression vector, from which a peptide or a library of peptides of diverse and/or random sequence may be expressed. A reduction in reporter gene expression (e.g. in the case of β-galactosidase a weakening of the blue colour) results from the presence of a peptide which disrupts the XRCC4/DNA ligase IV interaction, which interaction is required for transcriptional activation of the β-galactosidase gene. Where a test substance is not peptidyl and may not be expressed from encoding nucleic acid within a said third expression cassette, a similar system may be employed with the test substance supplied exogenously.

As noted, instead of using LexA and VP60, other similar combinations of proteins which together form a functional transcriptional activator may be used, such as the GAL4 DNA binding domain and the GAL4 transcriptional activation domain.

When performing a two hybrid assay to look for substances which interfere with the interaction between two

polypeptides or peptides it may be preferred to use mammalian cells instead of yeast cells. The same principles apply and appropriate methods are well known to those skilled in the art.

The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 nM to 100 μM or more concentrations of putative inhibitor compound may be used, for example from 0.1 to 50 μM, such as about 10 μM. Greater concentrations may be used when a peptide is the test substance. Even a molecule with weak binding may be a useful lead compound for further investigation and development.

Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used.

Antibodies directed to the site of interaction in either protein form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage *et al.*, 1992,

Nature **357**: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimicks that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation

or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include

fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies may also be used in purifying and/or isolating a polypeptide or peptide according to the present invention, for instance following production of the polypeptide or peptide by expression from encoding nucleic acid therefor. Antibodies may be useful in a therapeutic context (which may include prophylaxis) to disrupt XRCC4/DNA ligase IV interaction with a view to inhibiting their activity. Antibodies can for instance be micro-injected into cells, e.g. at a tumour site. Antibodies may be employed in accordance with the present invention for other therapeutic and non-therapeutic purposes which are discussed elsewhere herein.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or

peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

A compound found to have the ability to affect XRCC4 and/or DNA ligase IV activity has therapeutic and other potential in a number of contexts, as discussed. For therapeutic treatment such a compound may be used in combination with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy. In such a case, the assay of the invention, when conducted *in vivo*, need not measure the degree of inhibition of binding or of modulation of DNA ligase IV activity caused by the compound being tested. Instead the effect on DNA repair, homologous recombination, cell viability, cell killing (e.g. in the presence and absence of radio- and/or chemo-therapy), retroviral integration, and so on, may be measured. It may be that such a modified assay is run in parallel with or subsequent to the main assay of the invention in order to confirm that any such effect is as a result of the inhibition of binding or interaction between XRCC4 and DNA ligase IV caused by said inhibitor compound and not merely a general toxic effect.

Remember that the inventors have found interaction between XRCC4 and DNA-Pkcs/Ku and affecting this interaction is a part of the various aspects of the present invention in analogous fashion to affecting the interaction between XRCC4 and DNA ligase IV. The present inventors' finding in this respect is confirmed by Leber *et al.*, "The XRCC4 gene product is a target for and interacts with the DNA-dependent protein kinase" *J. Biol. Chem* (1998) Jan. 16 **273** (3), 1794 - according to information available from the World Wide Web on 12 January 1998.

An agent identified using one or more primary screens (e.g. in a cell-free system) as having ability to bind XRCC4 and/or DNA ligase IV and/or modulate activity of XRCC4 and/or DNA ligase IV may be assessed further using one or more secondary screens. A secondary screen may involve testing for cellular radiosensitisation and/or sensitisation to radiomimetic drugs, testing for impairment of V(D)J recombination in a transfection assay, and/or testing for ability to potentiate homologous recombination-mediated gene targeting. This may be tested directly and/or using a transfection assay that gives a read-out only after homologous recombination has occurred, (e.g. involving co-transformation or co-transfection of a cellular system with two plasmids that must undergo homologous recombination to yield an active reporter gene (such as luciferase or green fluorescent protein), or homologous integration of tranfected DNA into the genome.

Following identification of a substance or agent which modulates or affects XRCC4 and/or DNA ligase IV activity, the substance or agent may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals, e.g. for any of the purposes discussed elsewhere herein.

As noted, the agent may be peptidyl, e.g. a peptide which includes a sequence as recited above, or may be a functional analogue of such a peptide.

As used herein, the expression "functional analogue" relates to peptide variants or organic compounds having the same functional activity as the peptide in question, which may interfere with the binding between XRCC4 and DNA ligase IV. Examples of such analogues include chemical compounds

which are modelled to resemble the three dimensional structure of the XRCC4 or DNA ligase IV domain in the contact area, and in particular the arrangement of the key amino acid residues as they appear in XRCC4 or DNA ligase IV.

In a further aspect, the present invention provides the use of the above substances in methods of designing or screening for mimetics of the substances.

Accordingly, the present invention provides a method of designing mimetics of XRCC4 or DNA ligase IV having the biological activity of DNA ligase IV or XRCC4 binding or inhibition, the activity of allosteric inhibition of DNA ligase IV or XRCC4 and/or the activity of modulating, e.g. inhibiting, XRCC4/DNA ligase IV interaction, said method comprising:

(i) analysing a substance having the biological activity to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

Suitable modelling techniques are known in the art. This includes the design of so-called "mimetics" which involves the study of the functional interactions fluorogenic oligonucleotide the molecules and the design of compounds which contain functional groups arranged in such a manner that they could reproduced those interactions.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary

canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target

property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

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Mimetics of this type together with their use in therapy form a further aspect of the invention.

The present invention further provides the use of a peptide which includes a sequence as disclosed, or a derivative, active portion, analogue, variant or mimetic, thereof able to bind XRCC4 or DNA ligase IV and/or modulate, e.g. inhibit, interaction between XRCC4 and DNA ligase IV and/or modulate, e.g inhibit, XRCC4 and/or DNA ligase IV activity, in screening for a substance able to bind DNA ligase IV and/or XRCC4, and/or modulate, e.g. inhibit, interaction between XRCC4 and DNA ligase IV, and/or inhibit XRCC4 and/or DNA ligase IV activity.

Generally, such a substance, e.g. inhibitor, according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologicaly acceptable excipients. As noted below, a composition according to the present invention may include in addition to an inhibitor compound as disclosed, one or more other molecules of therapeutic use, such as an anti-tumour agent.

The present invention extends in various aspects not only to a substance identified as a modulator of XRCC4 and DNA ligase IV interaction and/or XRCC4 or DNA ligase IV-mediated activity, property or pathway, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for a purpose discussed elsewhere herein, which may include preventative treatment, use of such a substance in manufacture of a composition for administration, e.g. for a purpose discussed elsewhere herein, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A substance according to the present invention such as an inhibitor of XRCC4 and DNA ligase IV interaction or binding may be provided for use in a method of treatment of the human or animal body by therapy which affects DNA repair or other XRCC4 or DNA ligase IV-mediated activity in cells, e.g. tumour cells. Other purposes of a method of treatment employing a substance in accordance with the present invention are dicussed elsewhere herein.

Thus the invention further provides a method of modulating DNA repair activity, particularly DSB end-joining, or other XRCC4 and/or DNA ligase IV-mediated activity, e.g. for a purpose discussed elsewhere herein, which includes administering an agent which modulates, inhibits or blocks the binding of XRCC4 to DNA ligase IV protein, such a method being useful in treatment where such modulation, inhibition or blocking is desirable.

The invention further provides a method of treatment which includes administering to a patient an agent which interferes with the binding of XRCC4 to DNA ligase IV. Exemplary purposes of such treatment are discussed elsewhere

herein.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule, mimetic or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline

solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Liposomes, particularly cationic liposomes, may be used in carrier formulations.

Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The agent may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells.

Targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they may be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector may targeted to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

The agent (e.g. small molecule, mimetic) may be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT, the former involving targeting the activator to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activator, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

An agent may be administered in a form which is inactive but which is converted to an active form in the body. For instance, the agent may be phosphorylated (e.g. to improve solubility) with the phosphate being cleaved to provide an active form of the agent in the body.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated, such as cancer, virus infection or any other condition in which a XRCC4 or DNA ligase IV-mediated effect is desirable.

Nucleic acid according to the present invention, encoding a polypeptide or peptide able to modulate, e.g. interfere with, XRCC4 and DNA ligase IV interaction or binding and/or induce or modulate activity or other XRCC4 or DNA ligase IV-mediated cellular pathway or function, may be used in methods of gene therapy, for instance in treatment of individuals, e.g. with the aim of preventing or curing (wholly or partially) a disorder or for another purpose as discussed elsewhere herein.

Vectors such as viral vectors have been used in the prior art to introduce nucleic acid into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of

the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

Receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells, is an example of a technique for specifically targeting nucleic acid to particular cells.

A polypeptide, peptide or other substance able to modlate or interfere with the interaction of the relevant polypeptide, peptide or other substance as disclosed herein, or a nucleic acid molecule encoding a peptidyl such molecule, may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

Further aspects of the present invention arise from the fact that the work described herein provides indication that

mammals including humans deficient in XRCC4 and/or DNA ligase IV will have immune deficiencies, heightened cancer predisposition, particularly lyphoreticular malignancies, and/or will be radiosensitive.

For example, a small but significant percentage of human patients have disastrously debilitating (sometimes fatal) reactions to standard clinical doses of radiation. This is unfortunate, particularly where alternative modes of (e.g.) cancer treatment are available. The present invention allows and provides for diagnosis of such radiosensitive patients.

Diagnosis of XRCC4 and/or DNA ligase IV deficiency, which may be reduced ability of the particular polypeptide of an individual to interact with the other, or another component of a DNA repair pathway, may be used in conjunction with similar analysis of activity, function or structural integrity of other components of DNA repair pathways, such as Ku70, Ku80, DNA-PKcs, etc.

A number of methods are known in the art for analysing biological samples from individuals to determine whether the individual carries an allele of a particular gene predisposing them to a particular disorder. The purpose of such analysis may be used for diagnosis or prognosis, and serve to detect the presence of an existing defect (e.g. radiosensitivity), to help identify the type of defect (e.g. a factor in a manifest clinical disorder, such as cancer), to assist a physician in determining the severity or likely course of a disorder and/or to optimise treatment of it. Alternatively, the methods can be used to detect alleles that are statistically associated with a susceptibility to a disorder in the future, e.g. cancer, identifying individuals who would benefit from regular screening to provide early diagnosis of the disorder, e.g. cancer.

For instance, oligonucleotides designed to hybridise to a region within the gene of interest may be used in diagnostic and prognostic screening.

Oligonucleotide probes or primers, as well as the full-length gene sequence (and mutants, alleles, variants and derivatives) are useful in screening a test sample containing nucleic acid for the presence of alleles, mutants and variants, especially those that confer susceptibility or predisposition to a particular disorder, including radiosensitivity and cancers, the probes hybridising with a target sequence from a sample obtained from the individual being tested. The conditions of the hybridisation can be controlled to minimise non-specific binding, and preferably stringent to moderately stringent hybridisation conditions are preferred. The skilled person is readily able to design such probes, label them and devise suitable conditions for the hybridisation reactions, assisted by textbooks such as Sambrook et al (1989) and Ausubel et al (1992).

Nucleic acid isolated and/or purified from one or more cells (e.g. human) or a nucleic acid library derived from nucleic acid isolated and/or purified from cells (e.g. a cDNA library derived from mRNA isolated from the cells), may be probed under conditions for selective hybridisation and/or subjected to a specific nucleic acid amplification reaction such as the polymerase chain reaction (PCR).

A method may include hybridisation of one or more (e.g. two) probes or primers to target nucleic acid. Where the nucleic acid is double-stranded DNA, hybridisation will generally be preceded by denaturation to produce single-stranded DNA. The hybridisation may be as part of a PCR procedure, or as part of a probing procedure not involving PCR. An example procedure would be a combination of PCR and low stringency hybridisation. A screening procedure, chosen from the many available to those skilled in the art, is used to identify successful hybridisation events and isolated

hybridised nucleic acid.

Binding of a probe to target nucleic acid (e.g. DNA) may be measured using any of a variety of techniques at the disposal of those skilled in the art. For instance, probes may be radioactively, fluorescently or enzymatically labelled. Other methods not employing labelling of probe include examination of restriction fragment length polymorphisms, amplification using PCR, RNAase cleavage and allele specific oligonucleotide probing.

Probing may employ the standard Southern blotting technique. For instance DNA may be extracted from cells and digested with different restriction enzymes. Restriction fragments may then be separated by electrophoresis on an agarose gel, before denaturation and transfer to a nitrocellulose filter. Labelled probe may be hybridised to the DNA fragments on the filter and binding determined. DNA for probing may be prepared from RNA preparations from cells.

Those skilled in the art are well able to employ suitable conditions of the desired stringency for selective hybridisation, taking into account factors such as oligonucleotide length and base composition, temperature and so on.

PCR techniques for the amplification of nucleic acid are described in US Patent No. 4,683,195. In general, such techniques require that sequence information from the ends of the target sequence is known to allow suitable forward and reverse oligonucleotide primers to be designed to be identical or similar to the polynucleotide sequence that is the target for the amplification. PCR comprises steps of denaturation of template nucleic acid (if double-stranded), annealing of primer to target, and polymerisation. The nucleic acid probed or used as template in the amplification reaction may be genomic DNA, cDNA or RNA. PCR can be used to amplify specific sequences from genomic DNA, specific RNA

sequences and cDNA transcribed from mRNA, bacteriophage or plasmid sequences. References for the general use of PCR techniques include Mullis et al, Cold Spring Harbor Symp. Quant. Biol., 51:263, (1987), Ehrlich (ed), PCR technology, Stockton Press, NY, 1989, Ehrlich et al, Science, 252:1643-1650, (1991), "PCR protocols; A Guide to Methods and Applications", Eds. Innis et al, Academic Press, New York, (1990).

On the basis of amino acid sequence information, oligonucleotide probes or primers may be designed, taking into account the degeneracy of the genetic code, and, where appropriate, codon usage of the organism from the candidate nucleic acid is derived. An oligonucleotide for use in nucleic acid amplification may have about 10 or fewer codons (e.g. 6, 7 or 8), i.e. be about 30 or fewer nucleotides in length (e.g. 18, 21 or 24). Generally specific primers are upwards of 14 nucleotides in length, but not more than 18-20. Those skilled in the art are well versed in the design of primers for use processes such as PCR.

A further aspect of the present invention provides an oligonucleotide or polynucleotide fragment of XRCC4 or DNA ligase IV corresponding to part of the gene coding sequence or a complementary sequence, in particular for use in a method of obtaining and/or screening nucleic acid. The sequences referred to above may be modified by addition, substitution, insertion or deletion of one or more nucleotides, but preferably without abolition of ability to hybridise selectively with the relevant gene sequence, that is wherein the degree of homology of the oligonucleotide or polynucleotide with the sequence given is sufficiently high.

In some preferred embodiments, oligonucleotides according to the present invention that are fragments of the relevant gene sequence, in wild-type form or in the form of any allele associated with susceptibility to cancer or other

disorder, are at least about 10 nucleotides in length, more preferably at least about 15 nucleotides in length, more preferably at least about 20 nucleotides in length. Such fragments themselves individually represent aspects of the present invention. Fragments and other oligonucleotides may be used as primers or probes as discussed but may also be generated (e.g. by PCR) in methods concerned with determining the presence in a test sample of a sequence indicative of susceptibility to cancer or other disorder.

Preferred probes or primers according to certain embodiments of this aspect of the present invention are designed to hybridise with and/or amplify a fragment of the relevant sequence (e.g. XRCC4 or DNA ligase IV) including any residue mutation at which is associated with cancer susceptibility.

A number of methods are known in the art for analysing biological samples from individuals to determine whether the individual carries a gene allele with a mutation predisposing them to disease. The purpose of such analysis may be used for diagnosis or prognosis, and serve to detect the presence of, e.g., an existing cancer, to help identify the type of cancer, to assist a physician in determining the severity or likely course of the cancer and/or to optimise treatment of it. The methods may be used to detect alleles that are statistically associated with a susceptibility to cancer or other disorder in the future, e.g. early onset cancer, identifying individuals who would benefit from regular screening to provide early diagnosis of the disorder.

Broadly, the methods divide into those screening for the presence of nucleic acid sequences and those that rely on detecting the presence or absence of polypeptide. The methods make use of biological samples from individuals that are suspected of contain the nucleic acid sequences or

polypeptide. Examples of biological samples include blood, plasma, serum, tissue samples, tumour samples, saliva and urine.

Exemplary approaches for detecting nucleic acid or polypeptides include:

(a) comparing the sequence of nucleic acid in the sample with a XRCC4 and/or DNA ligase IV nucleic acid sequence to determine whether the sample from the patient contains one or more mutations, e.g. in a particular region, such as a region which interacts with counterpart DNA ligase IV or XRCC4, as the case may be, or other component of a DNA repair pathway, or, particularly in the case of DNA ligase IV, a catalytic region, or,

(b) determining the presence in a sample of a XRCC4 and/or DNA ligase polypeptide encoded by and, if present, determining whether the polypeptide includes a region corresponding to wild-type, and/or is mutated in such a region; or,

(c) using DNA fingerprinting to compare the restriction pattern produced when a restriction enzyme cuts a sample of nucleic acid from the patient with the restriction pattern obtained from a particular region corresponding to that for the normal gene or from known mutations thereof; or,

(d) using a specific binding member capable of binding to a nucleic acid sequence (either a normal sequence or a known mutated sequence) encoding a particular polypeptide fragment, the specific binding member comprising nucleic acid hybridisable with the relevant sequence, or substances comprising an antibody domain with specificity for a native or mutated polypeptide fragment nucleic acid sequence or the polypeptide encoded by it, the specific binding member being labelled so that binding of the specific binding member to its binding partner is detectable; or,

(e) using PCR involving one or more primers based on

the relevant normal or mutated gene sequence to screen for normal or mutant sequences within a particular region of the gene in a sample.

A "specific binding pair" in such a context may comprise a specific binding member (sbm) and a binding partner (bp) which have a particular specificity for each other and which in normal conditions bind to each other in preference to other molecules. Examples of specific binding pairs are antigens and antibodies (see above), molecules and receptors and complementary nucleotide sequences. The skilled person will be able to think of many other examples and they do not need to be listed here. Further, the term "specific binding pair" is also applicable where either or both of the specific binding member and the binding partner comprise a part of a larger molecule. In embodiments in which the specific binding pair are nucleic acid sequences, they will be of a length to hybridise to each other under the conditions of the assay, preferably greater than 10 nucleotides long, more preferably greater than 15 or 20 nucleotides long.

In most embodiments for screening for susceptibility alleles, the relevant nucleic acid (e.g. encoding XRCC4 and/or DNA ligase IV) in the sample will initially be amplified, e.g. using PCR, to increase the amount of the analyte as compared to other sequences present in the sample. This allows the target sequences to be detected with a high degree of sensitivity if they are present in the sample. This initial step may be avoided by using highly sensitive array techniques that are becoming increasingly important in the art.

To reiterate in further detail, the identification of biochemical activity and physiological function of XRCC4 and DNA ligase IV and particular regions thereof paves the way for aspects of the present invention to provide the use of

materials and methods, such as are disclosed and discussed above, for establishing the presence or absence in a test sample of an variant form of the gene, in particular an allele or variant specifically associated with cancer or other disorder such as radiosensitivity, as discussed. This may be for diagnosing a predisposition of an individual to a disorder. It may be for diagnosing a patient with a disorder as being associated with the gene.

This allows for planning of appropriate therapeutic and/or prophylactic treatment, permitting stream-lining of treatment by targeting those most likely to benefit.

A variant form of the gene may contain one or more insertions, deletions, substitutions and/or additions of one or more nucleotides compared with the wild-type sequence which may or may not disrupt the transcriptional activation function of the region examined herein. Differences at the nucleic acid level are not necessarily reflected by a difference in the amino acid sequence of the encoded polypeptide. However, a mutation or other difference in a gene may result in a frame-shift or stop codon, which could seriously affect the nature of the polypeptide produced, or a point mutation or gross mutational change to the encoded polypeptide, including insertion, deletion, substitution and/or addition of one or more amino acids or regions in the polypeptide, which may affect transcriptional activation.

There are various methods for determining the presence or absence in a test sample of a particular nucleic acid sequence, such as a sequence for XRCC4 or DNA ligase IV, or a fragment, mutant, variant or allele thereof.

Tests may be carried out on preparations containing genomic DNA, cDNA and/or mRNA. Testing cDNA or mRNA has the advantage of the complexity of the nucleic acid being reduced by the absence of intron sequences, but the possible disadvantage of extra time and effort being required in

making the preparations. RNA is more difficult to manipulate than DNA because of the wide-spread occurrence of RN'ases.

Nucleic acid in a test sample may be sequenced and the sequence compared with the relevant wild-tpye sequence to determine whether or not a difference is present. If so, the difference can be compared with known susceptibility alleles, to determine whether the test nucleic acid contains one or more of the variations indicated, or the difference can be investigated for association with the disorder of interest.

Since it will not generally be time- or labour-efficient to sequence all nucleic acid in a test sample or even the whole gene for XRCC4 or DNA ligase IV, a specific amplification reaction such as PCR using one or more pairs of primers may be employed to amplify the region of interest in the nucleic aci. The amplified nucleic acid may then be sequenced as above, and/or tested in any other way to determine the presence or absence of a particular feature. Nucleic acid for testing may be prepared from nucleic acid removed from cells or in a library using a variety of other techniques such as restriction enzyme digest and electrophoresis.

Nucleic acid may be screened using a variant- or allele-specific probe. Such a probe corresponds in sequence to a region of the relevant gene, or its complement, containing a sequence alteration known to be associated with susceptibility to cancer or other disorder of interest. Under suitably stringent conditions, specific hybridisation of such a probe to test nucleic acid is indicative of the presence of the sequence alteration in the test nucleic acid. For efficient screening purposes, more than one probe may be used on the same test sample.

Allele- or variant-specific oligonucleotides may similarly be used in PCR to specifically amplify particular

sequences if present in a test sample. Assessment of whether a PCR band contains a gene variant may be carried out in a number of ways familiar to those skilled in the art. The PCR product may for instance be treated in a way that enables one to display the mutation or polymorphism on a denaturing polyacrylamide DNA sequencing gel, with specific bands that are linked to the gene variants being selected.

SSCP heteroduplex analysis may be used for screening DNA fragments for sequence variants/mutations. It generally involves amplifying radiolabelled 100-300 bp fragments of the gene, diluting these products and denaturing at 95°C. The fragments are quick-cooled on ice so that the DNA remains in single stranded form. These single stranded fragments are run through acrylamide based gels. Differences in the sequence composition will cause the single stranded molecules to adopt difference conformations in this gel matrix making their mobility different from wild type fragments, thus allowing detecting of mutations in the fragments being analysed relative to a control fragment upon exposure of the gel to X-ray film.

Fragments with altered mobility/conformations may be directly excised from the gel and directly sequenced for mutation.

Sequencing of a PCR product may involve precipitation with isopropanol, resuspension and sequencing using a TaqFS+ Dye terminator sequencing kit. Extension products may be electrophoresed on an ABI 377 DNA sequencer and data analysed using Sequence Navigator software.

A further possible screening approach employs a PTT assay in which fragments are amplified with primers that contain the consensus Kozak initiation sequences and a T7 RNA polymerase promoter. These extra sequences are incorporated into the 5' primer such that they are in frame with the native coding sequence of the fragment being analysed. These PCR products are introduced into a coupled

transcription/translation system. This reaction allows the production of RNA from the fragment and translation of this RNA into a protein fragment. PCR products from controls make a protein product of a wild type size relative to the size of the fragment being analysed. If the PCR product analysed has a frame-shift or nonsense mutation, the assay will yield a truncated protein product relative to controls. The size of the truncated product is related to the position of the mutation, and the relative region of the gene from this patient may be sequenced to identify the truncating mutation.

An alternative or supplement to looking for the presence of variant sequences in a test sample is to look for the presence of the normal sequence, e.g. using a suitably specific oligonucleotide probe or primer.

Approaches which rely on hybridisation between a probe and test nucleic acid and subsequent detection of a mismatch may be employed. Under appropriate conditions (temperature, pH etc.), an oligonucleotide probe will hybridise with a sequence which is not entirely complementary. The degree of base-pairing between the two molecules will be sufficient for them to anneal despite a mis-match. Various approaches are well known in the art for detecting the presence of a mis-match between two annealing nucleic acid molecules.

For instance, RN'ase A cleaves at the site of a mis-match. Cleavage can be detected by electrophoresing test nucleic acid to which the relevant probe or probe has annealed and looking for smaller molecules (i.e. molecules with higher electrophoretic mobility) than the full length probe/test hybrid. Other approaches rely on the use of enzymes such as resolvases or endonucleases.

Thus, an oligonucleotide probe that has the sequence of a region of the normal gene (either sense or anti-sense strand) in which at least one mutation associated with, e.g., cancer susceptibility is known to occur, may be

annealed to test nucleic acid and the presence or absence of a mis-match determined. Detection of the presence of a mis-match may indicate the presence in the test nucleic acid of a mutation associated with, e.g., cancer susceptibility. On the other hand, an oligonucleotide probe that has the sequence of a region of the gene including a mutation associated with, e.g., cancer susceptibility may be annealed to test nucleic acid and the presence or absence of a mis-match determined. The presence of a mis-match may indicate that the nucleic acid in the test sample has the normal sequence. In either case, a battery of probes to different regions of the gene may be employed. Indeed, probes may be included with probes or other materials for other genes for stream-lined testing.

The presence of differences in sequence of nucleic acid molecules may be detected by means of restriction enzyme digestion, such as in a method of DNA fingerprinting where the restriction pattern produced when one or more restriction enzymes are used to cut a sample of nucleic acid is compared with the pattern obtained when a sample containing the normal gene or a variant or allele is digested with the same enzyme or enzymes.

A test sample of nucleic acid may be provided for example by extracting nucleic acid from cells, e.g. in saliva or preferably blood, or for pre-natal testing from the amnion, placenta or foetus itself.

Nucleic acid according to the present invention, such as a full-length coding sequence or oligonucleotide probe or primer, may be provided as part of a kit, e.g. in a suitable container such as a vial in which the contents are protected from the external environment. The kit may include instructions for use of the nucleic acid, e.g. in PCR and/or a method for determining the presence of nucleic acid of interest in a test sample. A kit wherein the nucleic acid

is intended for use in PCR may include one or more other reagents required for the reaction, such as polymerase, nucleosides, buffer solution etc. The nucleic acid may be labelled. A kit for use in determining the presence or absence of nucleic acid of interest may include one or more articles and/or reagents for performance of the method, such as means for providing the test sample itself, e.g. a swab for removing cells from the buccal cavity or a syringe for removing a blood sample (such components generally being sterile). In a further aspect, the present invention provides an apparatus for screening for XRCC4 and/or DNA ligase IV nucleic acid, the apparatus comprising storage means including the relevant gene nucleic acid sequence, or a fragment thereof, the stored sequence being used to compare the sequence of the test nucleic acid to determine the presence of mutations.

There are various methods for determining the presence or absence in a test sample of a particular polypeptide, such as a polypeptide including a fragment of XRCC4 or DNA ligase IV corresponding to a particular region involved in interaction with counterpart DNA ligase IV or XRCC4, as the case may be, involved in interaction with one or more other proteins or components of a DNA repair pathway, or having a particular biological activity, such as DNA ligase enzymatic activity.

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for one or more particular variants of the polypeptide, i.e. wild-type or a mutant, variant or allele thereof.

A sample may be tested for the presence of a binding partner for a specific binding member such as an antibody (or mixture of antibodies), specific for the polypeptide.

In such cases, the sample may be tested by being

contacted with a specific binding member such as an antibody under appropriate conditions for specific binding, before binding is determined, for instance using a reporter system as discussed. Where a panel of antibodies is used, different reporting labels may be employed for each antibody so that binding of each can be determined.

A specific binding member such as an antibody may be used to isolate and/or purify its binding partner polypeptide from a test sample, to allow for sequence and/or biochemical analysis of the polypeptide to determine whether it has the sequence and/or properties of the polypeptide of interest, or if it is a mutant or variant form. Amino acid sequence is routine in the art using automated sequencing machines.

Protein may be detected using Western blots, and also Far-Western blots in which a non-antibody protein is used. For instance, XRCC4 could be used to determine the presence of DNA ligase IV, and vice versa. Immunoprecipitation, radio-immunoassay, ELISA and other standard approaches in the art may be employed, using antibodies and other appropriate specific binding agents.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures discussed already above.

*EXAMPLE 1*

*DETERMINATION OF BIOLOGICAL ACTIVITY OF XRCC4*

*Generation of antisera that recognise XRCC4*

With the aim of gaining insights into the mechanism of

XRCC4 action, it was decided to try to characterise the human protein biochemically. Towards this end, full-length human XRCC4 and the C-terminal region of XRCC4 comprising residues 201-344 were expressed in *Escherichia coli* as hexa-histidine-tagged proteins. After purification to homogeneity, each antigen was then used to raise polyclonal antisera in rabbits.

In the course of these studies, we observed that recombinant full-length XRCC4 runs anomolously upon SDS-PAGE, with an apparent molecular mass of ~55 kDa, which is considerably larger than the predicted molecular weight of 38 kDa. Untagged and His-tagged versions of XRCC4 were found to behave similarly. Although the reason for this is currently unclear, this might reflect the fact that XRCC4 contains an unusually large proportion of glutamic acid amino acid residues, increasing the negative charge of the protein. One possible result of this would be a net decrease in the amount of SDS bound to the protein which would decrease the mobility of XRCC4 upon SDS-PAGE analysis.

Western blot analyses revealed that each of the anti-XRCC4 antisera raised was capable of recognising less than 1 ng of recombinant XRCC4 protein. To establish whether these antisera are capable of detecting endogenous XRCC4 in mammalian cell lysates, crude HeLa cell nuclear extracts were subjected to SDS-PAGE followed by Western immunoblot analysis.

Importantly, each antiserum, but none of the pre-immune sera, was found to recognise a HeLa cell protein of 55-60 kDa, which is in good agreement with the size of recombinant XRCC4. In addition, each immune serum also detects several other polypeptides weakly. Although the identities of these are not established, some may correspond to alternative forms of XRCC4 or its proteolytic degradation products. For instance, one band in particular is likely to represent a N-terminal XRCC4 proteolytic product because it is recognised

by all sera raised against the full-length protein but not by serum SJ5 that was raised against the XRCC4 C-terminal region. Interestingly, despite the high degree of sequence conservation between XRCC4 in rodents and humans (Li *et al.*, 1995), we have been unable to detect XRCC4 in extracts of mouse or hamster cells by direct Western blotting using these antibodies. This could in part reflect low immunological cross-reactivity between the human and rodent proteins. However, given the evolutionary conservation of XRCC4, the model that we currently favour is that, as is the case for other DNA DSB repair factors, such as Ku and DNA-PKcs (Blunt *et al.*, 1995; Finnie *et al.*, 1995; Danska *et al.*, 1996), XRCC4 is expressed at much lower levels in rodent cells than in human cells (also, see below).

To enhance further the specificity of anti-XRCC4 antiserum SJ4B, this was subjected to immuno-affinity chromatography using XRCC4 that had been attached covalently to Sepharose beads. Significantly, whereas the crude serum recognises a number of polypeptides in HeLa whole cell extracts in addition to full-length XRCC4, much of the reactivity towards the other proteins is recovered in the flow-through fractions, resulting in the affinity-purified antibody material (eluate) having improved specificity and selectivity as compared to the unfractionated serum.

*XRCC4 is a nuclear phosphoprotein and serves as an effective substrate for DNA-PK in vitro*

As a first step towards establishing the biochemical function of XRCC4, we decided to try to determine its sub-cellular localisation. Nuclear and cytosolic fractions were prepared from HeLa cells and were subjected to Western blot analysis using affinity-purified XRCC4 antibody SJ4B. The integrity of the fractions was established by also probing with antisera against Sp1 which is located predominantly in the nuclear fraction.

Notably, these studies revealed that XRCC4 is present in the nuclear extract, with low amounts being detectable in the cytosolic fraction. These data therefore reveal that XRCC4 is a nuclear protein and are consistent with models in which XRCC4 serves as part of a DNA DSB repair apparatus, for instance as illustrated in Figure 5.

In this model (which is proposed without in any way limiting the nature or scope of any aspect of the present invention or embodiment thereof), Ku binds to the free DNA ends and recruits DNA-PKcs, activating the kinase catalytic function of the latter. A DNA ligase IV/XRCC4 complex is then recruited to the DNA DSB. One or more additional components may be involved: some possibilities are indicated by means of question marks. Active DNA-PK may also trigger DNA damage signalling events or may phosphorylate other DNA DSB repair components, such as XRCC4 as has been demonstrated by the inventors, thus regulating their activity. The stoichiometry of the XRCC4-DNA ligase IV complex is for the purpose of illustration only. XRCC4 may interact with DNA ligase IV anywhere within residues 550-884 of human DNA ligase IV, e.g. at or between the BRCT domains.

During the course of the above studies, we observed that HeLa XRCC4 reproducibly migrates more slowly than recombinant XRCC4 on SDS-PAGE, suggesting that human XRCC4 is modified post-translationally. To determine whether this reflects XRCC4 phosphorylation, HeLa nuclear extract was either mock-treated, treated with λ protein phosphatase, or was treated with λ phosphatase in the presence of phosphatase inhibitors.

Significantly, Western analysis of these samples revealed that λ phosphatase increases the SDS-PAGE mobility of HeLa XRCC4 so that it is now equivalent to that of the recombinant protein, whereas this effect is abrogated by phosphatase inhibitors. These data therefore reveal that

XRCC4 is phosphorylated to high stoichiometry in HeLa cell extracts and suggest that this modification is used to modulate XRCC4 activity *in vivo*.

In light of this, and because cells deficient in XRCC4 have very similar phenotypes to those defective in components of DNA-PK, we tested whether DNA-PK is able to phosphorylate XRCC4 *in vitro*. XRCC4 indeed serves as an effective substrate for DNA-dependent phosphorylation by DNA-PK, so DNA-PK may control XRCC4 activity within the cell.

*Endogenous XRCC4 appears to be complexed with another protein(s)*

There are various ways in which XRCC4 might function in DNA DSB repair and V(D)J recombination.

One possibility the inventors considered is that it interacts directly with DNA and plays a role in repairing DNA damage or signalling its presence to the cell. However, we have been unable to detect binding of recombinant XRCC4 to various DNA species in electrophoretic mobility shift assays. Furthermore, when HeLa nuclear extracts are passed through DNA-agarose columns under salt concentrations in which many DNA binding proteins are retained, the majority of endogenous XRCC4 flows through.

These data therefore argue that XRCC4 does not bind avidly to DNA.

Another possible role for XRCC4 considered by the inventors is for it to interact with another component of the DNA DSB repair apparatus. As an approach to address this idea, we investigated the biochemical fractionation of XRCC4 and other known and potential DNA DSB repair factors upon gel-filtration chromatography on Superose-6. To disrupt possible non-specific protein-protein associations, such experiments were performed under stringent conditions of 1 M NaCl.

These studies revealed that recombinant untagged XRCC4 elutes in a manner consistent with a mass of just over 66 kDa, which is larger than the predicted XRCC4 monomer molecular weight (Li *et al.*, 1995) and its apparent mass as determined by SDS-PAGE. This suggests either that XRCC4 is a monomeric protein with shape characteristics causing it to behave anomolously upon gel-filtration, or exists in solution as a multimer, most likely a dimer.

Most significantly, gel-filtration analysis of HeLa nuclear extract in the presence of 1 M NaCl reveals that endogenous XRCC4 fractionates in a manner consistent with a molecular mass of around 200 kDa, which is markedly higher than that for recombinant XRCC4. These data therefore suggest strongly that HeLa XRCC4 is associated with another protein(s). We took the same set of gel-filtration fractions tested above for XRCC4 and examined them for the presence of Ku, DNA-$PK_{cs}$, and DNA ligases I, III and IV.

Significantly, although some overlap was evident in each case, the XRCC4 elution profile did not parallel those exhibited for DNA ligase I, Ku or DNA-$PK_{cs}$. Thus, ligase I peaked at ~150 kDa which is slightly larger than the predicted monomer molecular weight of 1-2 kDA, DNA-$PK_{cs}$ (465 kDA) eluted at around 200 kDa which may indicate that the tertiary structure of DNA-PK is disrupted under these conditions, and Ku elution peaked at ~150 kDa, consistent with the predicted size of a Ku70/Ku80 heterodimer.

In marked contrast, the elution profile of XRCC4 was found to be virtually identical to those of DNA ligases III and IV. These results therefore raised the possibility that XRCC4 exists in stable association with either DNA ligase III or IV.

*HeLa cell XRCC4 co-immunoprecipitates with DNA ligase IV*

To further test for possible interactions between XRCC4

and the factors described above, we immunoprecipitated XRCC4 from its peak gel-filtration fractions in the presence of 1 M NaCl and 50 µg/ml ethidium bromide (to abolish non-specific interaction mediated via DNA), and tested the resulting precipitated material for the presence of Ku, DNA-PK $_{cs}$, and ligases I, III, and IV. Significantly, Western immunoblot analyses revealed that DNA-$PK_{cs}$, Ku, and DNA ligase I that were present in the XRCC4 fractions did not co-immunoprecipitate with XRCC4, confirming that XRCC4 does not interact stably with any of these factors under these assay conditions. Note, however, that as discussed elsewhere herein, the inventors have established that DNA-PKcs/Ku is able to interact with XRCC4 under other conditions. They have shown also that it phosphorylates XRCC4, which of course requires some interaction between the proteins. This is confirmed by Leber *et al.*, "The XRCC4 gene product is a target for and interacts with the DNA-dependent protein kinase" *J. Biol. Chem* (1998) Jan. 16 **273** (3), 1794 - according to information available from the World Wide Web on 12 January 1998.

To assay for possible interactions between XRCC4 and a DNA ligase enzyme, we employed the fact that mammalian DNA ligases form covalently-linked adenylate complexes (Tomkinson *et al.*, 1991; Wei *et al.*, 1995; Danska *et al.*, 1996; Robins and Lindahl, 1996). When the XRCC4-containing gel-filtration fraction was incubated with [$\alpha$-$^{32}$P]-ATP and was then examined by SDS-PAGE followed by autoradiography, adenylated proteins of approximately 120 kDa and 100 kDa were detected, which correspond to DNA ligase I and a mixture of DNA ligases III and IV, respectively.

To see whether any of these ligases associate with XRCC4, unlabelled extract was incubated with pre-immune or anti-XRCC4 antisera in the presence of 1 M NaCl then, after stringent washing, the immunoprecipitated material was incubated with [$\alpha$-$^{32}$P]-ATP and tested for radioactively-

labelled adenylated proteins.

Significantly, these studies revealed that an adenylated protein species of ~100 kDa, corresponding to DNA ligase III and/or IV is immunoprecipitated efficiently by the affinity purified XRCC4 antiserum but not by pre-immune sera. By contrast, the adenylated species corresponding to DNA ligase I is not recovered. Importantly, and consistent with the fact that the adenylate moiety of adenylated-DNA ligase complexes is discharged in the presence of ligatable polynucleotide substrates, the radiolabel associated with the XRCC4-precipitated material is lost upon incubation in the presence of DNA that has been nicked by treatment with DNase I.

To rule out the possibility that the immunoprecipitated ligase was being recognised directly by the anti-XRCC4 antiserum, we performed parallel immunoprecipitation reactions on extracts derived from the hamster cell lines K1 and XR-1, which contain and lack XRCC4 protein, respectively. Importantly, the ~100 kDa adenylated ligase species is recovered from K1 extracts but not from XR-1 extracts. These data therefore reveal that the ligase is not recognised by the antiserum directly and, instead, is immunoprecipitated via its association with XRCC4.

Taken together, the above results reveal that XRCC4 forms a tight salt-stable interaction with DNA ligase III and/or DNA ligase IV. To establish which of these two enzymes is associated with XRCC4, we took advantage of the fact that ligases III and IV have different abilities to join single-strand breaks in polynucleotide substrates containing one DNA strand and one RNA strand. Thus, whereas DNA ligase III can catalyse joining in both oligo(rA)•poly(dT) and oligo(dT)•poly(rA) substrates, ligase IV is only able to mediate joining of the latter (Robins, 1996).

In light of this, we performed adenylation assays on material immunoprecipitated with XRCC4 and then incubated the labelled immunoprecipitates with either oligo(dT)•poly(rA) or oligo(rA)•poly(dT). Notably, only oligo(dT)•poly(rA) resulted in dissociation of the adenylate group from the ligase that is immunoprecipitated with XRCC4.

These results therefore suggest strongly that XRCC4 interacts tightly and specifically with DNA ligase IV but not with DNA ligase III.

*XRCC4 and ligase IV co-purify extensively*

To confirm the interaction between XRCC4 and DNA ligase IV, and to gain insight into what proportion of the two proteins exists in this complex, we purified DNA ligase IV using established protocols (Robins, 1996) and tested for the presence of ligase IV and XRCC4 by quantitative Western immunoblot analyses at each chromatographic stage.

Fractions collected from the chromatographic columns were analysed on SDS-polyacrylamide gels and specific binding of antibodies was detected by immunoblots. The amount of specified protein in each fraction was quantitated from densitometric scans of the gels. The amount of each protein in analysed fractions as a proportion of its total amount was plotted for the samples separated by gel chromatography fractionation (Figure 1A), followed by a Mono S column (Figure 1B).

As demonstrated previously, we observed that DNA ligases III and IV co-elute during gel filtration chromatography (Figure 1A) but are resolved from one another by chromatography on Mono-S (Figure 1B). Significantly, XRCC4 tracks with ligase IV throughout these procedures but, in contrast, becomes separated from DNA ligase III at the Mono-S chromatography step (Figures 1A and 1B). Furthermore, XRCC4 is present even in more highly purified samples of DNA ligase IV generated via subsequent

chromatography on Mono Q, and immunoblot analyses of near homogenous ligase IV preparations that we had prepared previously demonstrates the existence of XRCC4. Samples further purified on a Mono Q column (Robins, 1996) were analysed on a 10% SDS-polyacrylamide gel by immunoblots testing for the presence of XRCC4 or DNA ligase IV. Molecular sizes were estimated from the migration of Kaleidoscope pre-stained markers.

In additional studies, we have also observed that XRCC4 and DNA ligase IV co-purify on phenyl-Sepharose. Indeed, short of incubation with harsh ionic detergents, we have yet to find a procedure to separate these two proteins.

Interestingly, the XRCC4 that co-purifies with ligase IV corresponds to the phosphorylated form of the protein as evidenced by its SDS-PAGE mobility and by the fact that this mobility is increased by phosphatase treatment.

Finally, it is particularly noteworthy that XRCC4 and ligase IV co-purify almost quantitatively with one another, and that no free pools of either factor are evident (for example, see Figures 1A and 1B). This therefore suggests that XRCC4 and DNA ligase IV are present at similar levels in the cell and that virtually all of each polypeptide exists in a complex with its partner.

*XRCC4 interacts with the C-terminal portion of DNA ligase IV that comprises two BRCT homology domains*

To gain insights into the basis for the highly specific binding of DNA ligase IV to XRCC4, we decided to try to determine which region(s) of ligase IV are involved in this interaction.

DNA ligase I, III and IV display high levels of sequence similarity to one another within a section that has been defined as the core ligase catalytic domain (Wei *et al.*, 1995). In addition, each ligase possesses discrete N- and/or C-terminal extensions that have been proposed to

confer unique properties on the three enzymes. Significantly, although the C-terminal extensions of DNA ligases III and IV show very little homology with one another at the primary sequence level, they possess one and two copies, respectively, of the recently identified BRCT homology domain (Koonin *et al.*, 1996; Callebaut and Mornon, 1997); see Discussion below).

With the aim of finding which region(s) of ligase IV interacts with XRCC4, we divided the ligase IV polypeptide into three portions - an N-terminal region (corresponding to amino acid residues 1-198) that exhibits homology with ligase I and III, a central region (residues 199-549) that shows highest levels of homology with ligase I and III and which contains the ligase catalytic site, and a C-terminal region (residues 550-844) that contains the two BRCT homology domains. After transcribing and translating the three regions separately *in vitro*, these were tested for an ability to bind to Sepharose beads or to Sepharose beads containing covalently-attached XRCC4. Samples of LigIV(1-198), Lig IV(199-549), Lig IV(550-844) and luciferase were applied to XRCC4-Sepharose beads or negative control beads (ON) and unbound proteins collected (FT). After washes with either 0.1 M and 1.0 M NaCl, bound proteins were eluted with gel loading buffer (SDS). After SDS-PAGE, the [$^{35}$S]methionine-labelled fragments were detected by autoradiography.

The N-terminal and central fragments of DNA ligase IV fail to bind detectably to the XRCC4-beads, as is the case for the luciferase protein that was employed as a control. In marked contrast, the ligase IV C-terminal fragment is retained almost quantitatively on the XRCC4-beads but not on control beads lacking XRCC4. Moreover, the binding of the C-terminal portion of ligase IV to XRCC4 appears to be very strong, as evidenced by the fact that the ligase IV C-

terminal region is not eluted by washing at 1 M NaCl and is only recovered following addition of the ionic detergent SDS.

Finally, to further address the specificity of the above interaction, we assessed whether XRCC4-containing beads could be used to purify the C-terminal region of ligase IV from crude bacterial lysates. To do this, an unfractionated extract of *E. coli* expressing this region (LIGIV (550-844)) at fairly low levels was incubated with XRCC4-containing Sepharose beads and unbound proteins collected. Bound material was then eluted with step-wise increases in salt concentrations, followed by a final elution in the presence of gel loading buffer, SDS.

Strikingly, as shown by total Coomassie-blue protein staining of an SDS-polyacrylamide gel containing these fractions, this procedure results in the C-terminal region of ligase IV being purified to virtual homogeneity in a single step. The identity of this polypeptide as the ligase IV C-terminus was confirmed by Western blot analyses, and this protein was not retained by Sepharose beads alone.

Taken together, these results attest to the extreme strength and specificity of the interaction between the ligase IV C-terminal region and XRCC4.

*MATERIALS AND METHODS*

*Enzymes, antibodies and DNA*

pET-30b and pQE-30 were obtained from Novagen and Qiagen, respectively. Purified mouse monoclonal MRGS.His antibody (Qiagen) that recognises pQE-30-derived His-tagged proteins was used as per manufacturer's instructions. Ku70, Ku80, and DNA-$PK_{cs}$ antisera were used as described previously (Hartley *et al.*, 1995; Finnie *et al.*, 1996). Antibodies against ligase I (TL5), ligase III (TL25) and ligase IV (TL18) were used as described by (Lasko *et al.*, 1990; Robins

and Lindahl, 1996). Antigen-antibody complexes were detected by enhanced chemi-luminescence (Amersham) according to the manufacturer's instructions. HeLa nuclear extract was obtained from Computer Cell Culture Centre, Mons, Belgium. All plasmid constructs were verified by automated DNA sequencing.

*Expression and purification of XRCC4 derivatives*

To generate recombinant untagged XRCC4, the full-length *XRCC4* coding region was amplified from pBlueScript containing the human *XRCC4* gene by PCR and inserted into pET-30a (Novagen) digested with *Nde* I/*Sal* I such that the N-terminal His/S tags are not present and so that the *XRCC4* stop codon prevents the addition of the C-terminal His-tag. For protein expression, BL21(DE3) cells harbouring the resulting plasmid (pET30XRCC4) were grown in 500 ml cultures of LB/kanamycin (50 µg/ml) to mid-log prior to induction with 0.4 mM IPTG for 4 h at 37°C. After lysing the collected cell pellet by sonication, 30.2 g of ammonium sulphate was added slowly per 100 ml of supernatant and incubated with stirring at 4°C for 30 min. After centrifugation, the pellet was resuspended in TED (50 mM Tris-HCl pH 7.5, 2 mM DTT and 1 mM EDTA) and dialysed extensively against TED. Protein was then loaded onto a heparin-Sepharose column pre-equilibrated with TED and protein was eluted with a linear gradient of 0 to 0.6 M NaCl. Fractions containing XRCC4 were pooled and dialysed against TED containing 1.0 M ammonium sulphate then were loaded onto a pre-equilibrated phenyl-Sepharose column. Proteins were eluted with a 100 ml linear gradient of 1.0 to 0 M $(NH_4)_2SO_4$. Fractions containing XRCC4, eluting at ~0.2 M $(NH_4)_2SO_4$, were pooled and dialysed against 50 mM Tris.HCl pH 7.5, 2 mM DTT, 1 mM EDTA and 10% (w/v) glycerol, and stored at -80 °C.

*Anti-XRCC4 antibody production and purification*

Regions of the *XRCC4* gene were amplified by PCR from pBlueScript containing the human *XRCC4* gene then were inserted in-frame downstream of the hexa-histidine (His) tag of pQE-30 (Qiagen, USA) and were expressed and purified according to the manufacturer's instructions from the soluble fraction of bacterial lysates. Antibodies against the soluble recombinant proteins were raised in rabbits using standard procedures (Harlow and Lane, 1988) and are available commercially from Serotec, UK. Western immunoblot analyses were performed as described previously (Harlow and Lane, 1988) and blots were developed by enhanced chemi-luminescence (Amersham). Recombinant His-tagged full-length XRCC4 was attached to Sulfolink Coupling Gel (Pierce, USA) and was used to immuno-affinity purify anti-XRCC4 antibodies from crude SJ4 serum as described previously (Lakin *et al.*, 1996).

*Phosphatase-treatment of HeLa cell extracts*

To analyse for XRCC4 phosphorylation, HeLa nuclear extract (50 µg) was treated with λ protein phosphatase (New England Biolabs) in the presence of 2 mM $MnCl_2$ and incubated for 30 min at 30°C prior to SDS-PAGE and Western blotting.

*Co-immunoprecipitations and ligase adenylylation assays*

XRCC4 was immunoprecipitated from HeLa nuclear extract using polyclonal anti-XRCC4 and a pre-immune control. Specifically HeLa nuclear extract was dialysed into Buffer D* (20 mM HEPES-KOH, 20% (w/v) glycerol, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM sodium metabisulphite and 0.1% NP-40) and then incubated with either pre-immune or immune serum for 1 h at 4°C in the presence of 50 µg/ml ethidium bromide to disrupt non-specific interactions (Lai and Herr, 1992). Immune complexes were bound to protein A Sepharose beads

(Pharmacia), followed by extensive washing with Buffer D* containing 0.15-1 M NaCl. The protein A Sepharose beads were finally washed in Buffer D* containing 0.15 M NaCl prior to analysis. The samples were then tested for the ability to form DNA ligase-adenylated complexes as described previously (Robins and Lindahl, 1996). The polynucleotide substrates oligodT•polyrA and oligorA•polydT were prepared as described (Tomkinson *et al.*, 1991). The reactivity of the enzyme-adenylate intermediates formed was examined by adding 0.8 µg of unlabelled oligodT•polyrA or oligorA•polydT for 1 hr at 30°C. The reactions were stopped by the addition of SDS sample buffer and adenylylated proteins detected by autoradiography following SDS-PAGE.

*Gel-filtration chromatography*

Total HeLa nuclear extract (6 mg protein) was dialysed extensively against Buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 mM DTT, 10% (w/v) glycerol) containing 1 M NaCl. The protein was then loaded onto a Superose 6 (Pharmacia) column (60 × 1.5 cm) pre-equilibrated with Buffer A containing 1 M NaCl. On an identical gel-filtration run, 0.2 mg of pure untagged recombinant XRCC4 was analysed in buffer A containing 1 M NaCl.

*Purification of DNA ligase IV from HeLa cells*

DNA ligase IV was purified from HeLa cells as described previously (Robins, 1996). Fractions collected from each of the columns were analysed by immunoblots with antibodies specific for DNA ligases III and IV and XRCC4.

*Expression of recombinant ligase IV derivatives*

For generation of recombinant ligase IV derivatives, fragments of the human ligase IV gene coding region were amplified by PCR from reverse transcribed HeLa RNA. Each PCR product included a *Bam* HI site at the 5' end and a stop

codon followed by a*Sal* I site at the 3' end, and after digestion were ligated into pET30b digested with *Bam* HI/*Sal* I. The 550-844 fragment of ligase IV was also cloned into pQE-30 and the resultant clone was expressed in *E. coli* M15(Rep4). For *in vitro* transcription and translation of ligase IV fragments, 1 µg of pET30LigIV(1-198), pET30LigIV(199-549), pET30LigIV(550-844) or a luciferase control (Promega) were transcribed *in vitro* and translated using the TnT rabbit reticulocyte lysate kit (Promega) according to the manufacturer's instructions. Resulting N-terminally His-tagged ligase IV products were purified by $Ni^{2+}$-NTA agarose chromatography.

Briefly, a 100 µl bed volume of Qiagen $Ni^{2+}$-NTA agarose was pre-equilibrated in wash buffer (50 mM Tris-HCl pH 7.5, 20 mM imidazole, 10% (w/v) glycerol and 0.5 M NaCl) prior to the addition of 20 µl of the crude [$^{35}$S]methionine-labelled *in vitro* translated ligase fragment. Unbound proteins were removed after low-speed centrifugation and the resin was washed 3 times with wash buffer to remove non-specifically bound proteins. Finally, ligase IV proteins were eluted with 100 µl of elution buffer consisting of 50 mM Tris-HCl pH 7.5, 100 mM imidazole and 10% (w/v) glycerol.

*Interaction assays between recombinant XRCC4 and ligase IV derivatives*

Full-length XRCC4 was immobilised on Sepharose-4B gel beads (Pharmacia) using the cyanogen bromide method according to the manufacturer's instructions. As a negative control, coupling was also performed without XRCC4 protein. A 30 µl bed volume of beads (with and without XRCC4) was pre-equilibrated with Binding Buffer (50 mM Tris-HCl pH 7.5, 2 mM DTT, 1 mM EDTA, 10% (w/v) glycerol, 0.1% NP-40 and 0.36 mg/ml BSA) before the addition of the His-tag purified *in vitro* translated ligase products or luciferase. Unbound

material was collected after centrifugation and the beads were washed with Binding Buffer containing 0.1 M NaCl and 1.0 M NaCl. The beads were resuspended in gel loading buffer and boiled in preparation for analysis by SDS-PAGE. Finally SDS-PAGE gels were fixed in 10% acetic acid for 30 min and dried, and labelled proteins were detected by autoradiography. The ability of the recombinant C-terminal fragment of ligase IV (residues 550-844) to bind XRCC4-Sepharose beads was tested as above, except that analysis was by Coomassie-brilliant blue staining and immunoblotting with the anti-ligase IV antibody.

*EXAMPLE 2*

*DETERMINATION OF BIOLOGICAL ACTIVITY OF DNA LIGASE IV*

*Identification of a second, hitherto uncharacterised, yeast DNA ligase*

Using a consensus sequence within the core catalytic domain of all published DNA ligases the present inventors searched through the recently fully sequenced *S. cerevisiae* genome (Goffeau *et al.*, 1996).

In addition to detecting *CDC9*, which encodes DNA ligase I, these searches identified an ORF (YOR005c) present on chromosome XV as a highly significant hit.

This ORF encodes a 944 amino acid residue polypeptide of predicted molecular mass of 109 kDa that exhibits extensive similarity (24 % identity; 43 % similarity) in its central region to the "core" ligase conserved domain of DNA ligase I (Figure 2).

Phylogenetic analyses of protein alignments over this region reveal that YOR005c is considerably more related to DNA ligases of eukaryotes and eukaryotic viruses than to those of prokaryotes, and in particular, is most closely related to human ligase IV. A phenogram was generated using the PHYLIP package with the aligned "core" conserved

sequence of each protein as designated in Figure 2 using the UPGMA method. Accession numbers are as follows: *A. thaliana* I (X97924); *C. albicans* (X95001); *C. elegans* I (Z73970); Fowlpox virus (U00761); *H. sapiens* I (M36067), III (X84740) and IV (X83441); *M. musculus* I (U04674); Rabbit fibroma virus (Z29716); *S. cerevisiae* I (X03246), IV (Z74913); *S. pombe* I (X05107); T7 bacteriophage (P00969); Vaccinia virus (X16512); *X. laevis* I (L43496).

Consistent with this, YOR005c shares an N-terminal extension with the mammalian enzymes that is lacking in prokaryotic DNA ligases. Furthermore, it possesses an additional C-terminal extension that is homologous throughout its length to that of mammalian ligase IV. We thus conclude that YOR005c encodes a homologue of mammalian DNA ligase IV and designate this locus *LIG4* .

*Disruption of LIG4 does not lead to marked hypersensitivity to a variety of DNA-damaging agents*

To study *LIG4* function, we inactivated this gene in the haploid yeast strain W303α by a one-step gene disruption.

Notably, resulting *lig4* mutants do not have readily observable growth defects when propagated at temperatures ranging from 18°C to 37°C. This contrasts markedly with *CDC9*, the gene encoding yeast ligase I, whose disruption results in lethality due to an inability to progress through S-phase (Johnston and Nasmyth, 1978). It is thus concluded that *LIG4* does not play an essential role in DNA replication, and that yeast ligase I is the only DNA ligase required for this process.

Next, we tested whether *lig4* mutant yeast are defective in any of the predominant DNA repair pathways by assessing their sensitivity to killing by various DNA damaging agents. Notably, *lig4* mutant strains are not hypersensitive to DNA damage induced by exposure to ultraviolet radiation, showing that it is not essential in nucleotide excision repair. In

addition, strains disrupted for *LIG4* are not hypersensitive a range of concentrations of the radiomimetic drug, methyl methanesulfonate (MMS) in the growth medium. Finally, *lig4* mutant yeasts also do not display significantly elevated sensitivity to killing by ionising radiation at a range of doses (0 - 45 kRad). Unlike *rad52* mutants, *lig4* mutant yeast are as resistant to ionising radiation as parental strains: *lig4* mutants were not significantly more sensitive even at high doses (up to 45 kRad) (Figure 3A).

Since radiation-induced DNA double-strand-breaks (DSBs) are repaired primarily by homologous recombination in *S. cerevisiae*, these data suggest that *LIG4* is not essential for the latter process. Consistent with this, we have found that the efficiency of homologous recombination-mediated targeted integration into various loci in the yeast genome is indistinguishable between wild-type and *lig4* mutant strains.

*LIG4 functions in the Ku-dependent NHEJ pathway of DNA double-strand break repair*

In *S. cerevisiae*, radiation-induced DNA double-strand breaks (DSBs) are repaired primarily by homologous recombination, which is mediated by genes in the *RAD52* epistasis group (Friedberg *et al.*, 1995). Thus, disruption of *RAD52* sensitises yeast cells to ionising radiation (Figure 3A). However, eukaryotic cells can also repair DNA DSBs by a second pathway, non-homologous end-joining (NHEJ), that utilises gene products distinct from those employed in homologous recombination. In both yeast and mammals, one of these components is the DNA-binding protein Ku, comprising subunits of ~70 kDa and ~80 kDa [Ku70 and Ku80 in mammals (Jackson and Jeggo, 1995); Yku70p/Hdf1p and Yku80p/Hdf2p in yeast (Feldmann and Winnacker, 1993; Boulton and Jackson, 1996a; 1996b; Feldmann *et al.*, 1996; Mages *et al.*, 1996; Milne *et al.*, 1996; Siede *et al.*, 1996; Tsukamoto *et al.*,

1996)]. NHEJ appears to be the predominant pathway for DSB repair in mammals but represents a minor pathway in yeast; consequently, disruption of *S. cerevisiae YKU70* or *YKU80* only results in significantly increased sensitivity to ionising radiation or MMS when homologous recombination is inoperative (Boulton and Jackson, 1996a; 1996b; Milne *et al.*, 1996; Siede *et al.*, 1996).

We have found that *lig4/rad52* double mutants are considerably more radiosensitive than are strains disrupted for *RAD52* alone (Figure 3B). As in the case for *YKU70* (mutant in the Ku70 subunit of yeast Ku), disruption of *LIG4* hypersensitises yeast to ionising radiation in *rad52* mutant backgrounds. Furthermore, *lig4/yku70/rad52* triple mutants are not appreciably more radiosensitive than are *yku70/rad52* double mutant or *lig4/rad52* double mutant strains, indicating that Lig4p and Yku70p function on the same *RAD52*-independent repair pathway. This provides indication that *LIG4* is involved in the repair of ionising radiation-induced DNA damage and that it functions in a *RAD52*-independent pathway.

Since the effects of disrupting *LIG4* are similar to those obtained by disrupting *YKU70* or *YKU80*, we assessed the radiosensitivity of *lig4/yku70/rad52* triple mutants. Such mutant strains are no more sensitive to ionising radiation than are the *lig4/rad52* or *yku70/rad52* mutant strains (Figure 3B).

Taken together, these data provided indication that Ku and *LIG4* function in the same DNA repair pathway.

Previous work has shown that Ku functions in DNA NHEJ and that this process can be measured through employing an *in vivo* plasmid repair assay (Boulton and Jackson, 1996a; 1996b; Milne *et al.*, 1996). In this assay, a yeast-*E. coli* shuttle plasmid pBTM116 (Boulton and Jackson, 1996a; 1996b)

is linearised by restriction enzyme digestion, then is introduced into *S. cerevisiae* by transformation. The plasmid contains the yeast selectable marker *TRP1*, the β-lactamase gene, the *ADH1* promoter and *Eco*RI and *Pst*I restriction sites. Since the plasmid must be recircularised to be propagated, the number of yeast transformant colonies obtained quantifies the ability of the strain to repair the plasmid. Furthermore, since the DNA DSB generated in these studies resides in a region that is not homologous to the yeast genome, homologous recombination is suppressed and repair operates predominantly via NHEJ.

We therefore analysed the ability of *lig4* mutant yeasts to repair pBTM116 after cleavage with various restriction endonucleases. Wild-type, *lig4*, *yku70* and *lig4/yku70* yeast strains were used. Cells for each strain were transformed in parallel with supercoiled pBTM116 or with an equivalent amount of pBTM116 that had been digested to completion with *Eco*RI (left panel of Figure 4) or *Pst*I (right panel of Figure 4). For each experiment, the value plotted is the number of transformants obtained with the linear plasmid expressed as a percentage of the number obtained for supercoiled DNA. Each experiment was repeated at least three times and, in each case, cells were plated and counted in duplicate.

As with strains disrupted for *YKU70* or *YKU80*, *lig4* mutant strains are severely impaired in plasmid NHEJ, and this is observed both with 5' or 3' overhanging DNA ends. Interestingly, these studies reveal that the effect of *lig4* mutations is less pronounced with 3' overhanging DNA ends than it is with 5' overhanging ends. Although other alternatives exist, it is possible that this reflects differences in the mechanisms by which the two types of DNA ends can be repaired or is due to differential sensitivities of the different end structures to nuclease attack. Notably, DNA repair is not impaired further in *yku70/lig4* double

mutant strains (Figure 4). In addition, although the precise reason for this effect is not known, as is the case for *yku70* or *yku80* mutants, we have found that *lig4* mutant yeasts have a slightly elevated ability to rejoin pBTM116 bearing blunt-ends.

Taken together, these results reveal that Lig4p plays a crucial role in the repair of plasmid molecules bearing cohesive DNA double-strand breaks *in vivo*. Secondly, they show that, although purified DNA ligase I (*CDC9*) has been shown to be capable of catalysing DSB joining *in vitro* (Tomkinson *et al.*, 1992), this enzyme does not play a major role in this pathway as assayed by *in vivo* plasmid DSB rejoining, and is unable to substitute efficiently for Lig4p in this process. Finally these results also show that Lig4p plays an important role in the Ku-dependent NHEJ pathway.

Although plasmid repair is reduced dramatically in *lig4* mutant strains, it is not abolished. To determine precisely the types of DNA repair events that are dependent or independent of *LIG4*, repaired plasmids were recovered and then analysed by restriction enzyme digestion and DNA sequencing. In the absence of functional *LIG4*, the cohesive DNA termini are repaired by an inefficient error-prone DNA repair pathway. The plasmid pBTM116 contains the *ADH1* promoter and some repair products were generated by the gap-repair process involving the chromosomal *ADH1* gene. Of the large number of plasmids recovered from parental strains, all had been repaired by direct ligation of the cohesive DNA termini, thus regenerating the restriction enzyme cleavage site (Boulton and Jackson, 1996a; 1996b). Plasmid repair products recovered from *yku70* or *lig4* mutant strains, however, were found to fall into several categories. Some of these corresponded to "gap repair" products which we have shown are generated via *RAD52*-dependent homologous recombination with yeast genomic DNA (sequence analyses

reveal that homologous recombination is employed in the generation of these products and their production is abolished by disruption of *RAD52*; Boulton and Jackson, 1996a; 1996b and data not shown). This therefore provides further evidence that *LIG4*, like *YKU70* and *YKU80*, does not play a crucial role in homologous recombination processes. In *yku70* or *yku80* mutant strains, virtually all of the residual repair products were found to have suffered deletions (Boulton and Jackson, 1996a; 1996b). In contrast, although many of the residual repair products generated in *lig4* mutants had also suffered deletion of terminal sequences, some were rejoined accurately.

Collectively, these results provide insights into the distinct roles performed by Lig4p and Ku in DNA NHEJ (see Discussion below).

*Lig4p, unlike Ku, does not appear to function in telomere length maintenance*

Telomeres occur at the ends of eukaryotic chromosomes, are structurally distinct, and have unusual replication intermediates for which it is unclear whether a distinct DNA ligase is necessary (Blackburn, 1991; Zakian, 1995; Lundblad and Wright, 1996). Recent work has demonstrated that Ku functions in telomere homeostasis, since disruption of either *YKU70* or *YKU80* results in a dramatic reduction in telomeric length (Boulton and Jackson, 1996b; Porter *et al.*, 1996).

Given that Lig4p and Ku function together in DNA NHEJ, we tested whether *LIG4* is involved in telomere length control.

To do this, yeast genomic DNA was digested with the restriction enzyme *Xho*I, which in wild-type strains produces a predominant telomeric fragment of ~1.3 kb that is detected by Southern hybridisation to an oligonucleotide probe that binds to the repetitive telomeric sequences, including ~400

bp of repeating ($C_{1-3}A$) sequence and is detected by Southern hybridisation using a radiolabelled poly $(GT)_{20}$ oligonucleotide. Notably, whereas disruption of *YKU70* results in telomeric shortening, loss of *LIG4* function has no detectable effect.

These data therefore reveal that, although Ku and Lig4p function together in DNA DSB repair, Ku but not Lig4p has an additional essential function in telomere length homeostasis.

*MATERIALS AND METHODS*

*Gene disruptions*

Full length *LIG4* was amplified by PCR with primers *LIG4*-1 and *LIG4*-2 (5' TCAGTAGTTGACTACGGGAAAGTCT 3' and 5' ATGATATCAGCACTAGATTCTATAC 3', respectively) using the Expand High Fidelity DNA polymerase (Boehringer Mannheim). After cloning into pGEM-T (Promega), the resultant plasmid was digested with *Eco*RI, treated with *Pfu* DNA polymerase and then digested with *Xba*I. The *HIS3* marker was inserted to replace the *LIG4* ORF between residues 289 and 592. The disruption fragment was excised with *Sph*I and *Spe*I and was used to transform the appropriate yeast strains to $His^+$. Gene disruption was verified by using *LIG4* and *HIS3* primers in PCR. Two *RAD52* disruption constructs were provided by D. Weaver and have *TRP1* and *URA3* selection respectively.

*Assessment of sensitivity to temperature and DNA damaging agents*

Aliquots (15 µl) of serial 5-fold dilutions of mid-log phase yeast cultures were spotted onto YPDA plates and were grown for 36 h at 30°C or 37 °C. Strains on one plate were exposed to 50 $J/m^2$ ultraviolet (UV-C) radiation (Stratalinker; Stratagene). On another plate, YPDA medium

contained 0.0025% methylmethanesulfonate. In other studies, *lig4* mutant strains did not display hypersensitivity to MMS (0.0005% and 0.005% in the growth medium) nor to UV-C (20 - 150 $J/m^2$).

*Ionising irradiation survival assays*

Three independent isolates of each strain were inoculated either into minimal media lacking the appropriate amino acid(s) or into YPDA and were grown overnight at 30°C. Cultures were diluted in sterile water to an $OD_{600nm}$ value equivalent to 1 x $10^7$ cells/ml and 1 ml aliquots were irradiated using a $^{137}Cs$ source at a dose of 0.18 kRad/min. Irradiated samples and unirradiated controls were then diluted and plated in duplicate using an automated spiral plater (Whitley) on YPDA or minimal media. Colony numbers were ascertained following incubation at 30°C for 3 - 4 days.

*Plasmid repair assay*

Plasmid repair assays were performed as described previously (Boulton and Jackson, 1996a; 1996b). Briefly, the yeast-*Escherichia coli* shuttle plasmid pBTM116 (2 - 5 µg), which contains *TRP1* for selection in yeast, was digested with the appropriate restriction enzyme to completion and the enzyme was inactivated by treatment at 65°C for 20 min. Linearised DNA was then used to transform yeast by the lithium acetate method (Ausubel *et al.*, 1987). Parallel transformations were performed with an equivalent amount of uncut plasmid to enable normalisation for differences in transformation efficiency. Diluted samples were plated in duplicate on minimal media lacking the appropriate amino acids, and colonies were counted following incubation at 30°C for 3 - 4 days. To analyse plasmid repair products, DNA from single yeast transformants was isolated via the Yeast DNA Isolation kit (Stratagene) and this was used to

transform *E.* coli XL1-Blue cells (Stratagene) to ampicillin resistance. Plasmid DNA was then isolated and was analysed by restriction enzyme digestion and by DNA sequencing.

*Yeast DNA extraction and analyses of telomeric DNA*

Genomic DNA from *S. cerevisiae* was isolated essentially as described (Ausubel *et al.*, 1987). For telomere analyses, 2 µg of genomic DNA was digested with 30 U of *Xho*I (Boehringer Mannheim) at 37°C overnight. The digested DNA was then separated on a 1.2 % agarose 1 x TAE gel and was transferred to Hybond Nfp+ membrane (Amersham) by capillary transfer in 20 x SSC as suggested by the manufacturer. Membranes were pre-hybridised in 0.5 M sodium phosphate, pH 7.2, 1% SDS and then hybridised with 3 ng/ml of $^{32}$P-end-labelled poly $(GT)_{20}$ oligonucleotide (specific activity of $>10^9$/µg) in a Church-based buffer (0.2 M sodium phosphate, pH 7.2, 1% BSA, 6% polyethyleneglycol 6000, 1% SDS) overnight at 62°C. Finally, membranes were washed twice at room temperature for 30 min in 0.2 M sodium phosphate, 0.1% SDS, then exposed to pre-flashed X-ray film at -70°C.

*DISCUSSION*

The inventors' work with XRCC4 indicates that it is a predominantly nuclear protein. Moreover, through a variety of approaches, we have demonstrated that XRCC4 mediates extremely tight and specific interactions with DNA ligase IV. For example, these two components co-immunoprecipitate highly specifically with one another from HeLa cell extracts, even in the presence of 1 M NaCl. Furthermore, such interactions are not abrogated by ethidium bromide, indicating that the interaction between XRCC4 and ligase IV is not mediated by a DNA intermediate. Indeed, we show that bacterially expressed XRCC4 and ligase IV also bind to one another tightly, revealing that their interaction is direct. In addition, XRCC4 and ligase IV co-purify over every

chromatographic fractionation procedure we have employed, including gel-filtration in the presence of 1 M NaCl, anion and cation exchange chromatography, and hydrophobic interaction chromatography. Indeed, so far we have only resolved these two proteins by the addition of harsh ionic detergents.

The fact that XRCC4 interacts tightly with ligase IV but not with the other DNA ligases that we have analysed has lead us to investigate the basis for this binding specificity. Notably, although all characterised mammalian DNA ligases contain a common highly related core catalytic region, each possesses unique N- and/or C-terminal extensions. We have found that it is the unique C-terminal domain and not the ligase catalytic region of ligase IV that interacts with XRCC4. Interestingly, this region of ligase IV contains two tandem copies of the weakly conserved BRCT homology domain (Koonin *et al.*, 1996; Callebaut and Mornon, 1997), leading one to speculate that it is one or both of these domains that mediate the interaction with XRCC4. BRCT domains also exist in a variety of other factors and are required for those factors to interact (Mackey *et al.*, 1997; Nash *et al.*, 1997), suggesting that the BRCT domain of one protein interacts with a BRCT domain of the other. Thus, in light of our work showing interaction between XRCC4 and DNA ligase IV it might be expected that XRCC4 would also possess one or more copies of the BRCT consensus. Although XRCC4 has not been identified as a BRCT domain-containing protein by previous analyses, we have used manual and computer-aided inspections of the XRCC4 sequence to reveal limited homologies to other BRCT domains, suggesting that XRCC4 might contain one or more divergent copies of this putative protein structural unit.

Given that XRCC4 clearly functions in DNA NHEJ, our data indicate that DNA ligase IV also plays a crucial role in this process. XRCC4 may serve as a molecular bridge to

target ligase IV to DNA DSBs, perhaps through XRCC4 also interacting with other components of the DNA NHEJ apparatus (Figure 5). In regard to such a putative bridging function for XRCC4, it is worthy of note that immunoprecipitation studies suggest that XRCC4 can interact with Ku and/or DNA-PK $_{CS}$, although these interactions only occur at low salt concentrations and hence are weak compared to those exhibited between XRCC4 and ligase IV. A possible physical linkage between XRCC4 and DNA-PK is attractive in light of the fact that we have shown that HeLa cell XRCC4 is a phospho-protein and is an effective substrate for DNA-PK *in vitro*. Since XRCC4 possesses a DNA-PK kinase consensus motif (Li *et al.*, 1995), mutation of this site may affect XRCC4 function *in vivo*.

Consistent with the proposal that ligase IV plays an important role in DNA DSB repair, we have identified a *S. cerevisiae* homologue of DNA ligase IV (displaying extensive sequence similarity along its length with mammalian DNA ligase IV) and have shown that inactivation of this factor debilitates DNA NHEJ in a manner that is epistatic with mutations in the yeast homologues of Ku70 and Ku80 (Boulton and Jackson, 1996; Boulton and Jackson, 1996; Teo and Jackson, 1997). By contrast, we find that yeast ligase IV does not appear to play an essential role in the repair of ultraviolet light-induced DNA damage nor in the repair of DNA DSBs by homologous recombination (Teo and Jackson, 1997). Taken together with the data on XRCC4, this provides indication that ligase IV is dedicated to DNA NHEJ and that this function is conserved throughout the eukaryotic kingdom.

The yeast gene, which we have designated *LIG4*, is not essential for DNA replication, *RAD52*-dependent homologous recombination nor the pathways of nucleotide excision repair

and base excision repair. Instead, we have shown that *LIG4* is specifically involved in the rejoining of DNA double-strand breaks by the process of DNA NHEJ, which does not demand homology between the two recombining DNA molecules and does not require *RAD52*. Notably, genetic epistasis experiments reveal that *LIG4* acts in the same DNA repair pathway as Ku, a nuclear protein that specifically recognises DNA strand breaks. We have thus identified a novel *S. cerevisiae* DNA ligase and have shown that it is involved specifically in the Ku-dependent NHEJ pathway of DNA DSB repair.

In light of this, and given that mutations in *YKU70* or *YKU80* result in dramatic telomeric shortening in yeast (Boulton *et al.*, 1996b; Porter *et al.*, 1996), we have also assessed the potential involvement of *LIG4* in telomere length homeostasis. Telomeres are the protein-DNA structures at the ends of eukaryotic chromosomes that ensure the complete replication of chromosome ends, protect these ends from degradation, and prevent chromosomal termini from activating DNA damage signalling pathways or engaging in fusion and recombination reactions with other loci [for reviews (Blackburn, 1991; Zakian, 1995; Lundblad and Wright, 1996)]. In most organisms, telomeres are composed of variable numbers of simple repeat sequences and, at least in *S. cerevisiae*, the length of these sequence arrays is maintained by a combination of telomerase activity and *RAD52*-dependent and -independent recombination. In yeast, deficiencies in Ku result in an approximately 70% reduction in the number of telomeric repeat sequences (Boulton *et al.*, 1996b; Porter *et al.*, 1996). Given that Ku binds to the ends of double-strand DNA (Mimori and Hardin, 1986; Paillard and Strauss, 1991), one possibility is that Ku may interact directly with telomeric DNA ends and potentiate telomere lengthening by protecting telomeric DNA termini from nucleases or by augmenting telomerase recruitment. An

alternative explanation is that the effect of Ku inactivation on telomere length is indirect - perhaps the DNA repair defects that are associated with Ku deficient yeasts result in changes in cell physiology that impinge indirectly on telomere length control. Although it is not possible at present for us to identify precisely how Ku affects telomere length, the fact that mutations in *LIG4* have essentially the same DNA repair defect as Ku but do not alter telomere length argues for a specific role for Ku in telomere homeostasis that is distinct from its activities in DNA DSB repair. In this regard, it will be of interest to see whether mutated derivatives of Ku can be generated that have no effect on DNA repair but do result in defective telomeric maintenance.

Yeast cells mutated in *LIG4* have pronounced defects in DNA NHEJ, showing that Lig4p plays a crucial role in this process that cannot be complemented efficiently by yeast DNA ligase I. Conversely, yeast *CDC9* and human DNA ligase I mutants are defective in DNA replication and, at least *in vitro*, this function is not performed efficiently by other enzymes. This indicates that yeast DNA ligases I and IV have distinct and largely separate cellular functions and cannot substitute effectively for one another. Thus, DNA ligase I plays a crucial role in DNA replication and also appears to seal single-strand DNA breaks that are the end-products of nucleotide- and base-excision repair, and moreover, is likely to complete recombination events between homologous duplex DNA molecules. There are also data suggesting that mammalian DNA ligase III is specialised towards particular functions. One splice variant (DNA ligase III-α) may operate in a separate pathway for base excision repair while another variant (DNA ligase III-β) has been implicated in meiotic recombination. Notably, there are no obvious homologues of mammalian DNA ligase II/ III in *S. cerevisiae*. However, sequence analyses (Fig 1; Colinas et *al.*, 1990; Kerr *et al.*,

1991; Husain *et al.*, 1995) reveal that these ligases are related more closely to DNA ligases encoded by cytoplasmic poxviruses than they are to DNA ligase I, suggesting that ligases II and III may have arisen fairly recently in vertebrate evolution. Interestingly, and largely consistent with the proposed functions for mammalian ligase III, inactivation of poxvirus DNA ligase does not affect viral DNA replication or recombination but renders the mutant virus more sensitive to DNA damage induced by UV or bleomycin (Colinas *et al.*, 1990; Kerr *et al.*, 1991). Collectively, these data suggest that DNA ligase I and perhaps DNA ligase II/III are involved predominantly in the rejoining of single-stranded nicks whereas DNA ligase IV is the major enzyme catalysing the joining of double-stranded breaks.

In light of these points, and given that *LIG4* functions in the highly evolutionarily conserved Ku-dependent NHEJ pathway, mammalian DNA ligase IV is indicated as having a key role in Ku-dependent DNA DSB rejoining. As is the case for Ku (reviewed in Jackson and Jeggo, 1995), deficiency in mammalian ligase IV may result in cellular radiosensitivity and an inability to rejoin site-specific V(D)J recombination intermediates.

Although the available data suggest diversification of function for the different eukaryotic DNA ligases, it is unclear whether this arises from intrinsic differences in catalytic activity or from differences conferred, for example, by the distinct C- and N-terminal extensions of the enzymes. At least *in vitro*, purified human DNA ligases I, III and IV show differing capacities to join single-stranded breaks in hybrid polynucleotide substrates (Arrand *et al.*, 1986; Tomkinson *et al.*, 1991; Robins and Lindahl, 1996). Furthermore, purified mammalian DNA ligases differ in their abilities to rejoin DNA DSBs. It is noteworthy, however, that in contrast with the available *in vivo* data, these

studies show that purified ligase I but no other mammalian DNA ligase is able to catalyse the joining of blunt DNA ends effectively *in vitro* (Arrand *et al.*, 1986; Tomkinson *et al.*, 1991; Tomkinson *et al.*, 1992; Robins and Lindahl, 1996). One possible explanation for this discrepancy between the *in vitro* and *in vivo* data is that at least some of the eukaryotic DNA ligases may not have high intrinsic affinity for DNA and, within the cell, are targeted to appropriate DNA lesions by accessory factors. Consistent with this is the identification herein of strong interaction between DNA ligase IV and XRCC4.

Inactivation of either yeast Ku or Lig4p both result in a similar dramatic reduction of NHEJ in the *in vivo* plasmid DNA DSB repair assay. Because of this and since the level of DNA repair does not fall further in yeast strains defective in both Ku and Lig4p, we conclude that these two factors function in the same illegitimate recombination pathway. However, it is apparent that Ku and Lig4p have distinct functions in DNA NHEJ, as evidenced by the different spectra of residual plasmid repair products that are generated in the respective mutant strains. Thus, whereas nearly all the residual plasmid repair products arising in *yku70* mutants suffer deletions, in *lig4* mutant strains these correspond to a mixture of deletion products and products generated by accurate DNA end-joining. Collectively, these results suggest that Ku may function in at least two ways to potentiate DNA repair. Firstly, it may protect exposed DNA ends from nuclease attack. Secondly, it might serve to specifically recruit Lig4p, directly or indirectly, to the sites of DNA damage, perhaps via the Lig4p C-terminal extension that is absent from DNA ligase I. Consequently, the phenotypes of strains defective in Ku or Lig4p can both be explained to result from an inability to target a ligase to DNA DSBs efficiently. In Ku deficient strains, the ready access of nucleases to the DNA ends may

lead to deletions in virtually all the residual NHEJ repair products, which presumably arise via inefficient DNA end joining by untargeted ligase I or Lig4p. In contrast, when Lig4p is absent, Ku is still able to protect the DNA ends and this can explain how some accurate repair can still occur - this presumably being mediated by DNA ligase I. However, the reduced repair kinetics in *lig4* mutant yeast may mean that, even in the presence of Ku, nucleases ultimately gain access to the DNA termini and lead deletions in a large proportion of the residual repair products. Consistent with the above model, we find that virtually all of the residual NHEJ products generated in *yku70/lig4* double mutants have sustained terminal deletions.

*Interaction between XRCC4 and Ku/DNA-PKcs complex*

XRCC4 interaction with DNA-PKcs/Ku was demonstrated by incubation of HeLa cell nuclear extract with anti-XRCC4 or pre-immune antiserum with purification of the resulting immunocomplexes by adsoprtion onto protein A-Sepharose, then analysis by Western immunoblotting.

Both DNA-PKcs and the two subunits of Ku are immunoprecipitated by the anti-XRCC4 antiserum but not the pre-immune serum.

In these studies, immunocomplexes were washed under relatively mild conditions of 0.25 M NaCl and 0.1% Nonidet-P40. However, when more stringent washes were employed (for example, in the presence of 1 M NaCl, 0.1% Nonidet-P40 and 50 µg/ml ethidium bromide) the interaction between XRCC4 and Ku/DNA-PKcs complex was abolished.

Taken together, these data reveal that although the interaction between Ku/DNA-PKcs and XRCC4 appears specific, it is relatively weak.

The interaction may be inhibited using appropriate agents, including peptide fragments of the respective proteins. Such agents may be identified and obtained using

assay methods and used in therapeutic and other contexts, as disclosed above.

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CLAIMS:

1. An assay method for a compound able to modulate the interaction or binding between XRCC4 and DNA ligase IV, or XRCC4 and DNA-$PK_{CS}$/Ku, or XRCC4, DNA ligase IV and DNA-$PK_{CS}$/Ku, the method including the steps of:

(a) bringing into contact a substance including XRCC4 or a peptide fragment of XRCC4 or a derivative, variant or analogue thereof able to bind DNA ligase IV or DNA-$PK_{CS}$/Ku, a substance including DNA ligase IV or a peptide fragment of DNA ligase IV or a variant, derivative or analogue thereof able to bind XRCC4 and/or including DNA-$PK_{CS}$/Ku or a peptide fragment of DNA-$PK_{CS}$/Ku or a variant, derivative or analogue thereof able to bind XRCC4, and a test compound, under conditions wherein, in the absence of said test compound being an inhibitor of interaction or binding between said substances, said substances interact or bind; and

(b) determining interaction or binding between said substances.

2. An assay method for a compound able to modulate interaction or binding between XRCC4 and DNA ligase IV or XRCC4 and DNA-$PK_{CS}$/Ku, or XRCC4, DNA ligase IV and DNA-$PK_{CS}$/Ku, the method including the steps of:

(a) bringing into contact a substance including XRCC4 or a peptide fragment of XRCC4 which interacts with DNA ligase IV or DNA-$PK_{CS}$/Ku, or a derivative, variant or analogue thereof which interacts with DNA ligase IV or DNA-$PK_{CS}$/Ku, or which includes DNA ligase IV or DNA-$PK_{CS}$/Ku or a peptide fragment of DNA ligase IV or DNA-$PK_{CS}$/Ku which interacts with XRCC4, or a derivative, variant or analogue thereof which interacts with XRCC4, and a test compound; and

(b) determining interaction between said substances and the test compound.

3. An assay method for a compound able to affect DNA ligase IV activity, the method including the steps of:

(a) bringing into contact DNA ligase IV and a test compound; and

(b) determining DNA ligase activity.

4. An assay method according to claim 3 wherein the DNA ligase activity is determined in the presence of XRCC4.

5. An assay method according to 3 or 4 wherein the activity of DNA ligase is determined by its adenylation or labelling by using an ATP analogue, or ability to join strands of DNA or DNA analogues.

6. An assay method including

(a) bringing into contact a substance which includes DNA-PKcs/Ku or suitable peptide fragments of DNA-PKcs/Ku or derivative, variant or analogue thereof able to phosphorylates XRCC4, a substance which includes XRCC4 or a peptide fragment of XRCC4 or a derivative, variant or analogue thereof including a site phosphorylated by DNA-PKcs, and a test compound; and

(b) determining phosphorylation at said site.

7. An agent capable of modulating interaction between XRCC4 and DNA ligase IV, or XRCC4 and DNA-$PK_{CS}$/Ku, or XRCC4 and DNA ligase IV and DNA-$PK_{CS}$/Ku, obtained using a method according to claim 1 or claim 2.

8. A peptide fragment of DNA ligase IV capable of modulating interaction between XRCC4 and DNA ligase IV, or XRCC4 and DNA ligase IV and DNA-$Pk_{cs}$/Ku.

9. A peptide according to claim 8 which has a sequence found in human DNA ligase IV between amino acid residues

591-676 using the numbering of Wei, *et al.*, (1995). *Mol. Cell. Biol.* **15**, 3206-3216.

10. A peptide according to claim 8 which has a sequence found in human DNA ligase IV between amino acid residues 728-844 using the numbering of Wei, *et al.*, (1995). *Mol. Cell. Biol.* **15**, 3206-3216.

11. A peptide according to claim 8 which has a sequence found in human XRCC4 between amino acid residues 677-727 using the numbering of Wei, *et al.*, (1995). *Mol. Cell. Biol.* **15**, 3206-3216.

12. A nucleic acid isolate encoding a peptide according to any one of claims 8 to 11.

13. A peptide fragment of XRCC4 capable of modulating interaction between XRCC4 and DNA ligase IV.

14. A nucleic acid isolate encoding a peptide according to claim 13.

15. An agent able to affect DNA ligase IV activity obtained using a method according to any one of claims 3 to 5.

16. An agent able to affect DNA-PKcs/Ku phosphorylation of XRCC4 obtained using a method according to claim 6.

17. An agent or peptide fragment or nucleic acid isolate according to any of claims 5 to 16 for use in a method of treatment by therapy involving modulating cellular DNA repair activity.

18. Use of an agent or peptide fragment or nucleic acid

isolate according to any of claims 5 to 16 in the manufacture of a medicament for modulating cellular DNA repair activity.

19. A method which includes, following obtaining a compound able to modulate the interaction or binding between XRCC4 and DNA ligase IV, or XRCC4 and DNA-$PK_{CS}$/Ku, or XRCC4 and DNA ligase IV and DNA-$PK_{CS}$/Ku, employing a method according to claim 1 or claim 2, formulating the compound into a composition including a pharmaceutically acceptable excipient.

20. A method which includes, following obtaining a compound able to modulate the interaction or binding between XRCC4 and DNA ligase IV, or XRCC4 and DNA-$PK_{CS}$/Ku, or XRCC4 and DNA ligase IV and DNA-$PK_{CS}$/Ku, employing a method according to claim 1 or claim 2, providing the compound to a cell to modulate cellular DNA repair activity.

21. A method according to claim 20 wherein the cell is not part of a human or animal body.

22. A method which includes, following obtaining a compound able to affect DNA ligase IV activity employing a method according to any one of claims 3 to 5, formulating the compound into a composition including a pharmaceutically acceptable excipient.

23. A method which includes, following obtaining a compound able to affect DNA ligase IV activity employing a method according to any one of claims 3 to 5, providing the compound to a cell to modulate cellular DNA repair activity.

24. A method according to claim 23 wherein the cell is not part of a human or animal body.

25. A method which includes, following obtaining a compound able to affect DNA-PKcs/Ku phosphorylation of XRCC4 employing a method according to claim 6, formulating the compound into a composition including a pharmaceutically acceptable excipient.

26. A method which includes, following obtaining a compound able to affect DNA-PKcs/Ku phosphorylation of XRCC4 employing a method according to claim 6, providing the compound to a cell to modulate cellular DNA repair activity.

27. A method according to claim 26 wherein the cell is not part of a human or animal body.

28. A method for screening an individual for a predisposition to a disorder in which DNA repair plays a role, the method including determining from a sample taken from the individual the presence or absence of a defect in XRCC4 and/or DNA ligase IV activity.

Fig.1A.

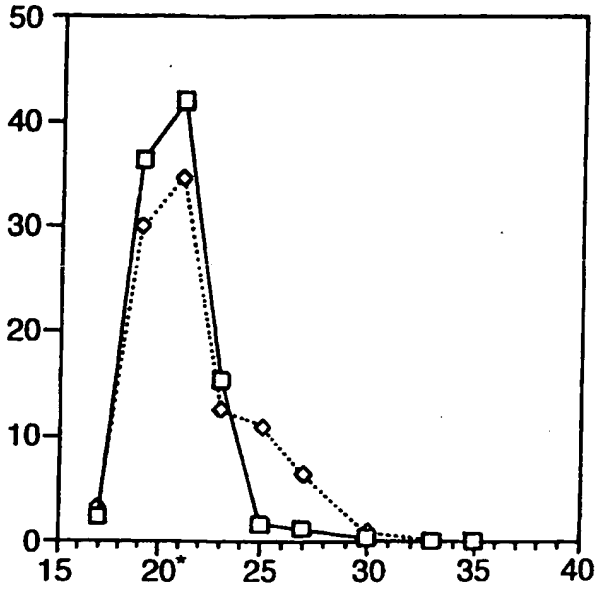
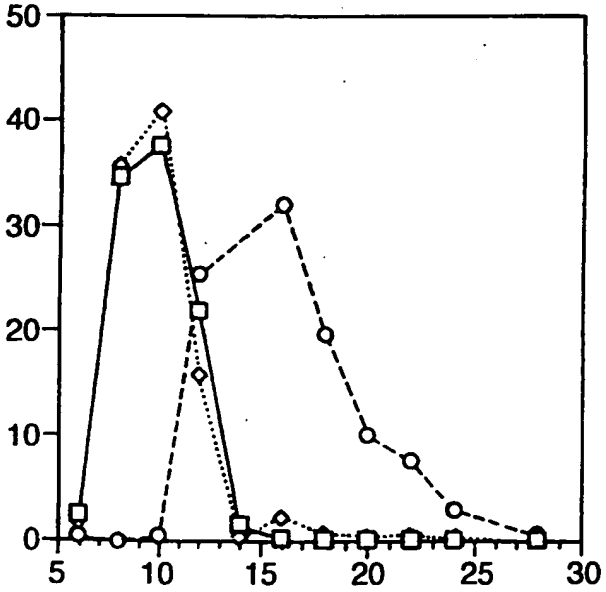



Fig.1B.



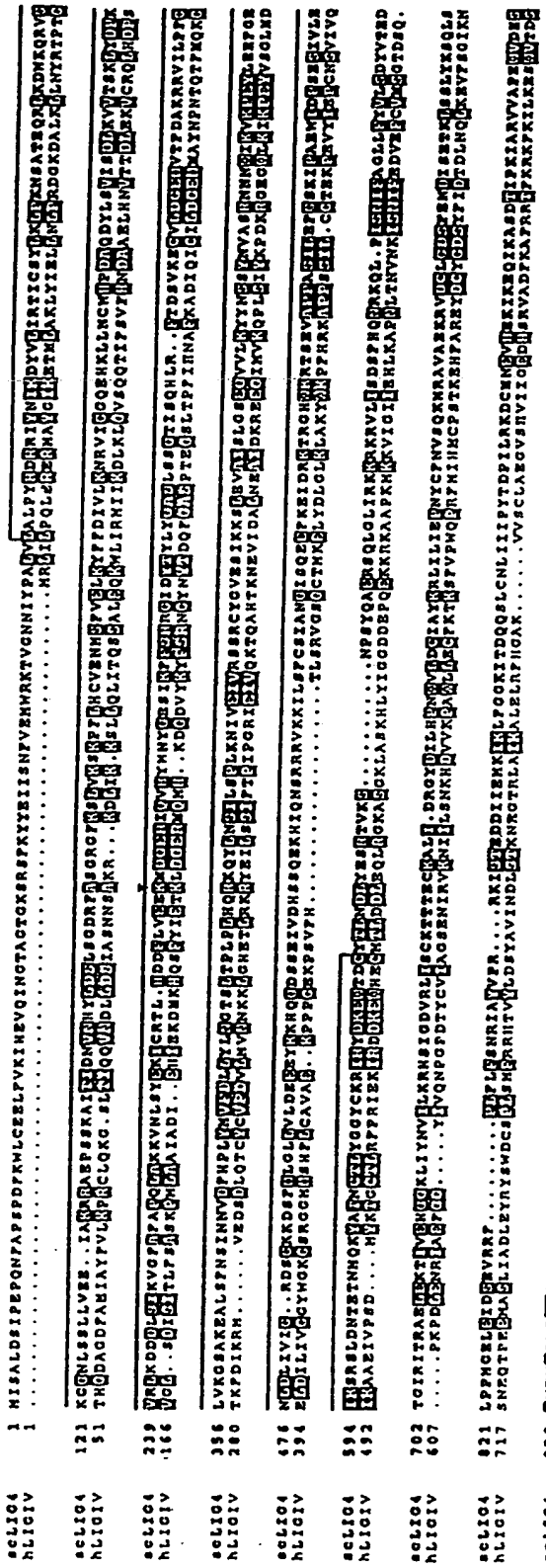
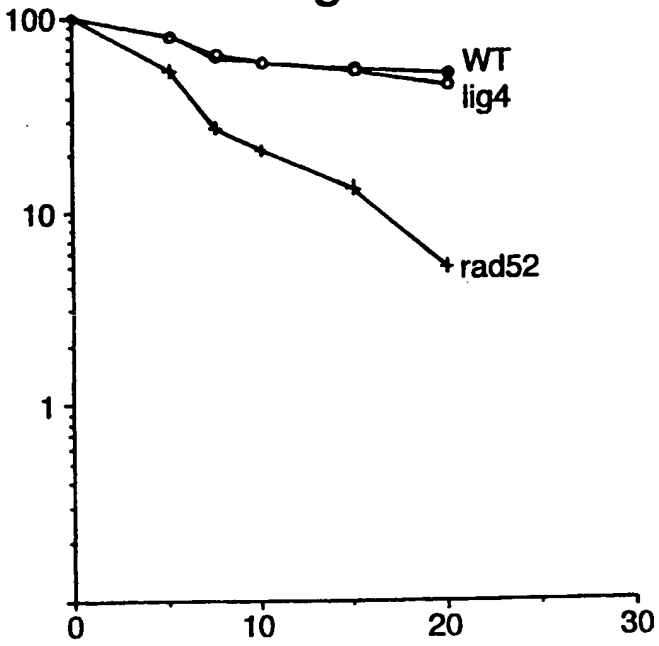


Figure 2

Fig.3A.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0
10
20
30
WT
lig4
rad52

Fig.3B.

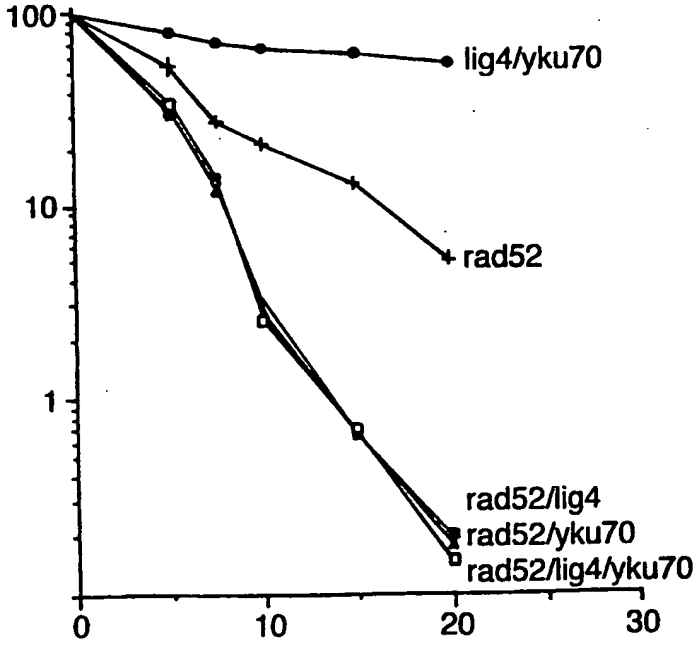
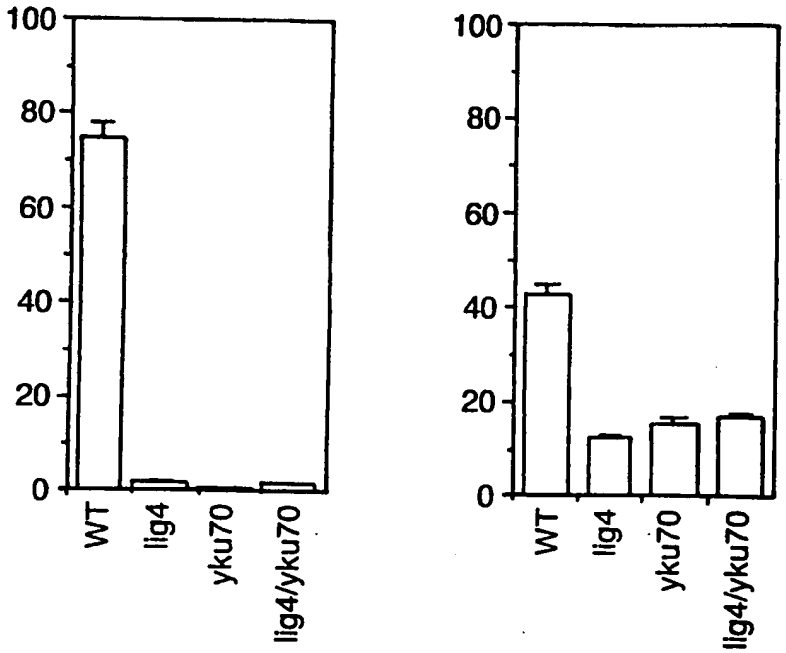

100
10
1
0
10
20
30
lig4/yku70
rad52
rad52/lig4
rad52/yku70
rad52/lig4/yku70

Fig.4.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60
40
20
0
WT
lig4
yku70
lig4/yku70
100
80
60
40
20
0
WT
lig4
yku70
lig4/yku70

Fig.5.

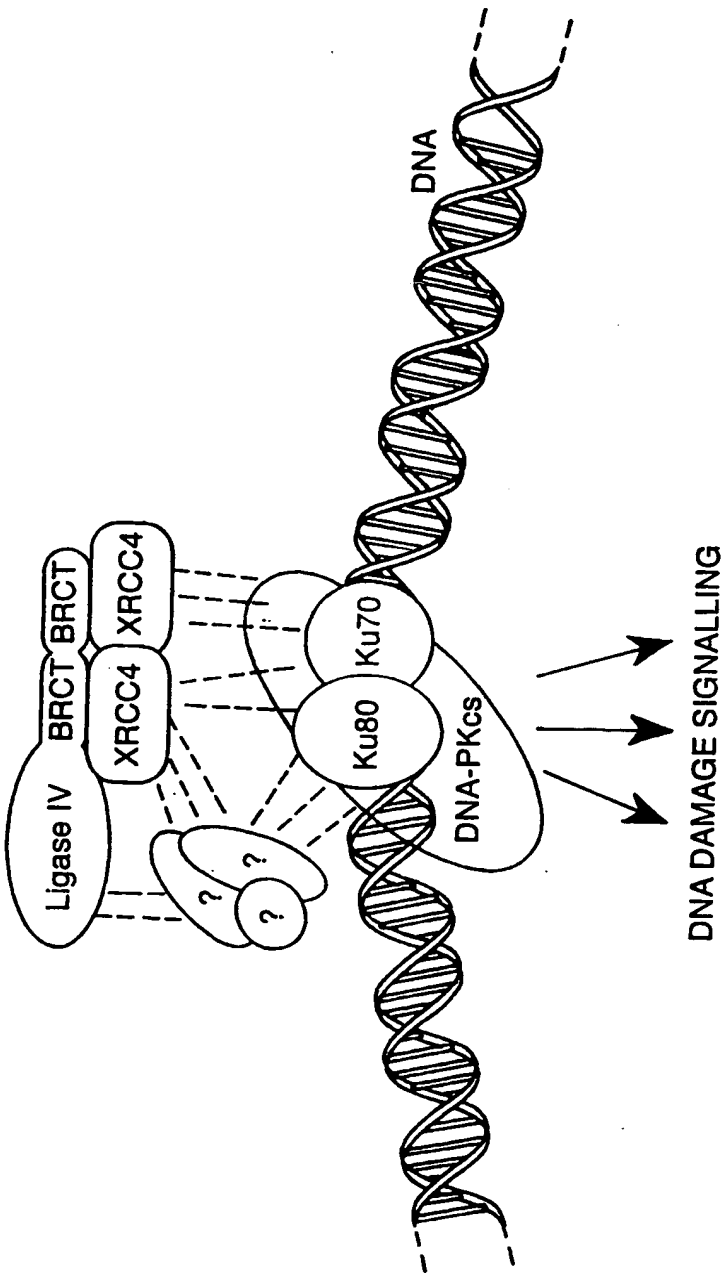
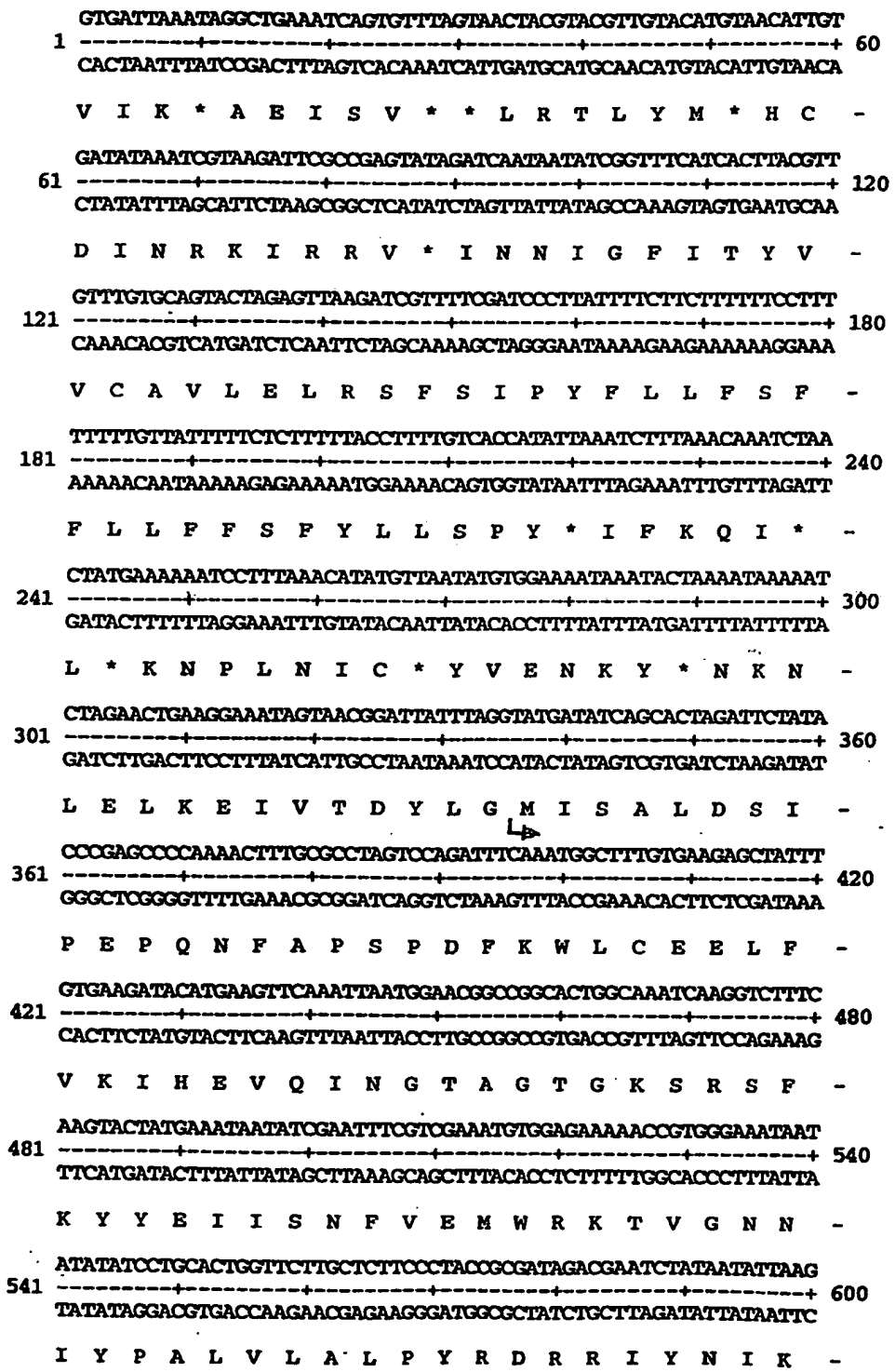

DNA
Ligase IV
BRCT
BRCT
XRCC4
XRCC4
Ku70
Ku80
DNA-PKcs
?
?
?
DNA DAMAGE SIGNALLING

Figure 6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V I K * A E I S V * * L R T L Y M * H C -
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61
120
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L * K N P L N I C * Y V E N K Y * N K N -
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301
360
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L E L K E I V T D Y L G M I S A L D S I -
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361
420
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421
480
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V K I H E V Q I N G T A G T G K S R S F -
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481
540
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K Y Y E I I S N F V E M W R K T V G N N -
ATATATCCTGCACTGGTTCTTGCTCTTCCCTACCGCGATAGACGAATCTATAATATTAAG
541
600
TATATAGGACGTGACCAAGAACGAGAAGGGATGGCGCTATCTGCTTAGATATTATAATTC
I Y P A L V L A L P Y R D R R I Y N I K -

Figure 6 (Continued)

```
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601 ---------+---------+---------+---------+---------+---------+ 660
    CTAATACATAATTATTCTTGATATACGAGAATGAACTTCAACGGTTTTTTAAGACGTTGT

    D  Y  V  L  I  R  T  I  C  S  Y  L  K  L  P  K  N  S  A  T  -

    GAGCAGCGGTTAAAAGATTGGAAACAGCGTGTCGGTAAAGGTGGGAATCTTTCTTCTCTT
661 ---------+---------+---------+---------+---------+---------+ 720
    CTCGTCGCCAATTTTCTAACCTTTGTCGCACAGCCATTTCCACCCTTAGAAAGAAGAGAA

    E  Q  R  L  K  D  W  K  Q  R  V  G  K  G  G  N  L  S  S  L  -

    CTTGTGGAAGAAATTGCTAAAAGAAGGGCTGAACCTAGCTCAAAAGCGATTACAATTGAT
721 ---------+---------+---------+---------+---------+---------+ 780
    GAACACCTTCTTTAACGATTTTCTTCCCGACTTGGATCGAGTTTTCGCTAATGTTAACTA

    L  V  E  E  I  A  K  R  R  A  E  P  S  S  K  A  I  T  I  D  -

    AACGTCAATCACTATCTGGATAGTTTGAGTGGAGACAGGTTCGCTTCCGGACGAGGATTT
781 ---------+---------+---------+---------+---------+---------+ 840
    TTGCAGTTAGTGATAGACCTATCAAACTCACCTCTGTCCAAGCGAAGGCCTGCTCCTAAA

    N  V  N  H  Y  L  D  S  L  S  G  D  R  F  A  S  G  R  G  F  -

    AAGAGTCTTGTCAAGTCCAAACCTTTCCTGCACTGTGTGGAGAATATGAGTTTCGTCGAA
841 ---------+---------+---------+---------+---------+---------+ 900
    TTCTCAGAACAGTTCAGGTTTGGAAAGGACGTGACACACCTCTTATACTCAAAGCAGCTT

    K  S  L  V  K  S  K  P  F  L  H  C  V  E  N  M  S  F  V  E  -

    TTAAAATACTTCTTTGATATCGTGCTTAAAAATAGAGTAATAGGAGGTCAAGAGCACAAA
901 ---------+---------+---------+---------+---------+---------+ 960
    AATTTTATGAAGAAACTATAGCACGAATTTTTATCTCATTATCCTCCAGTTCTCGTGTTT

    L  K  Y  F  F  D  I  V  L  K  N  R  V  I  G  G  Q  E  H  K  -

    TTGCTAAACTGCTGGCATCCTGATGCTCAGGATTATCTTAGCGTGATATCTGATTTAAAG
961 ---------+---------+---------+---------+---------+---------+ 1020
    AACGATTTGACGACCGTAGGACTACGAGTCCTAATAGAATCGCACTATAGACTAAATTTC

    L  L  N  C  W  H  P  D  A  Q  D  Y  L  S  V  I  S  D  L  K  -

    GTGGTAACTTCAAAACTTTATGATCCAAAAGTTCGTCTAAAGGATGATGATTTGAGTATA
1021 ---------+---------+---------+---------+---------+---------+ 1080
    CACCATTGAAGTTTTGAAATACTAGGTTTTCAAGCAGATTTCCTACTACTAAACTCATAT

    V  V  T  S  K  L  Y  D  P  K  V  R  L  K  D  D  D  L  S  I  -

    AAAGTTGGCTTTGCATTCGCCCCCCAATTAGCCAAAAAAGTGAATCTTTCTTATGAGAAA
1081 ---------+---------+---------+---------+---------+---------+ 1140
    TTTCAACCGAAACGTAAGCGGGGGGGTTAATCGGTTTTTTCACTTAGAAAGAATACTCTTT

    K  V  G  F  A  F  A  P  Q  L  A  K  K  V  N  L  S  Y  E  K  -

    ATATGCCGTACACTACATGATGATTTTTTGGTAGAAGAAAAAATGGATGGAGAACGAATT
1141 ---------+---------+---------+---------+---------+---------+ 1200
    TATACGGCATGTGATGTACTACTAAAAAACCATCTTCTTTTTTACCTACCTCTTGCTTAA

    I  C  R  T  L  H  D  D  F  L  V  E  E  K  M  D  G  E  R  I  -

    CAAGTTCATTATATGAATTATGGTGAATCCATAAAATTTTTTAGTAGACGGGGCATCGAC
1201 ---------+---------+---------+---------+---------+---------+ 1260
    GTTCAAGTAATATACTTAATACCACTTAGGTATTTTAAAAAATCATCTGCCCCGTAGCTG

    Q  V  H  Y  M  N  Y  G  E  S  I  K  F  F  S  R  R  G  I  D  -
```

Figure 6 (Continued)

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1261  ---------+---------+---------+---------+---------+---------+ 1320
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      Y  T  Y  L  Y  G  A  S  L  S  S  G  T  I  S  Q  H  L  R  F  -

      ACAGATAGTGTTAAAGAATGTGTTTTAGATGGAGAAATGGTGACGTTTGATGCAAAAAGA
1321  ---------+---------+---------+---------+---------+---------+ 1380
      TGTCTATCACAATTTCTTACACAAAATCTACCTCTTTACCACTGCAAACTACGTTTTTCT

      T  D  S  V  K  E  C  V  L  D  G  E  M  V  T  F  D  A  K  R  -

      CGGGTGATTCTTCCATTCGGTCTTGTTAAAGGAAGTGCAAAGGAAGCGCTATCTTTTAAT
1381  ---------+---------+---------+---------+---------+---------+ 1440
      GCCCACTAAGAAGGTAAGCCAGAACAATTTCCTTCACGTTTCCTTCGCGATAGAAAATTA

      R  V  I  L  P  F  G  L  V  K  G  S  A  K  E  A  L  S  F  N  -

      AGTATAAATAATGTTGACTTTCACCCCTTATATATGGTGTTTGATCTGTTATACCTGAAT
1441  ---------+---------+---------+---------+---------+---------+ 1500
      TCATATTTATTACAACTGAAAGTGGGGAATATATACCACAAACTAGACAATATGGACTTA

      S  I  N  N  V  D  F  H  P  L  Y  M  V  F  D  L  L  Y  L  N  -

      GGGACTTCGTTGACACCATTACCCCTTCATCAAAGGAAGCAATATCTGAACAGCATTTTA
1501  ---------+---------+---------+---------+---------+---------+ 1560
      CCCTGAAGCAACTGTGGTAATGGGGAAGTAGTTTCCTTCGTTATAGACTTGTCGTAAAAT

      G  T  S  L  T  P  L  P  L  H  Q  R  K  Q  Y  L  N  S  I  L  -

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1561  ---------+---------+---------+---------+---------+---------+ 1620
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      S  P  L  K  N  I  V  E  I  V  R  S  S  R  C  Y  G  V  E  S  -

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1621  ---------+---------+---------+---------+---------+---------+ 1680
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      I  K  K  S  L  E  V  A  I  S  L  G  S  E  G  V  V  L  K  Y  -

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1681  ---------+---------+---------+---------+---------+---------+ 1740
      ATATTAAGTTCAATATTACAGCGGTCAGCTTTGTTGTTGACCTAGTTCCATTTTGGACTT

      Y  N  S  S  Y  N  V  A  S  R  N  N  N  W  I  K  V  K  P  E  -

      TATTTGGAGGAATTTGGAGAGAATTTAGACTTAATAGTAATAGGCAGAGATTCTGGGAAA
1741  ---------+---------+---------+---------+---------+---------+ 1800
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      Y  L  E  E  F  G  E  N  L  D  L  I  V  I  G  R  D  S  G  K  -

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1801  ---------+---------+---------+---------+---------+---------+ 1860
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1861  ---------+---------+---------+---------+---------+---------+ 1920
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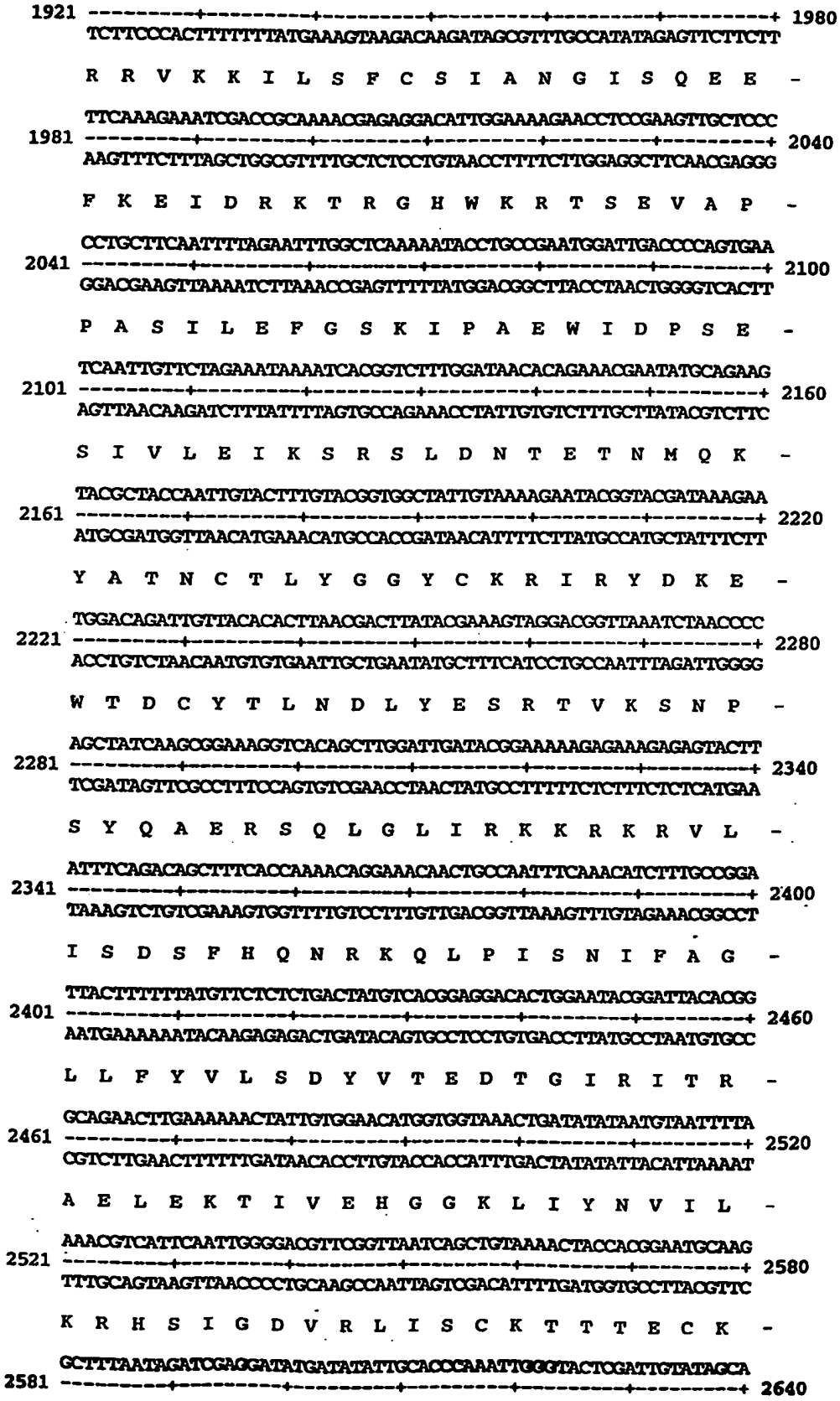

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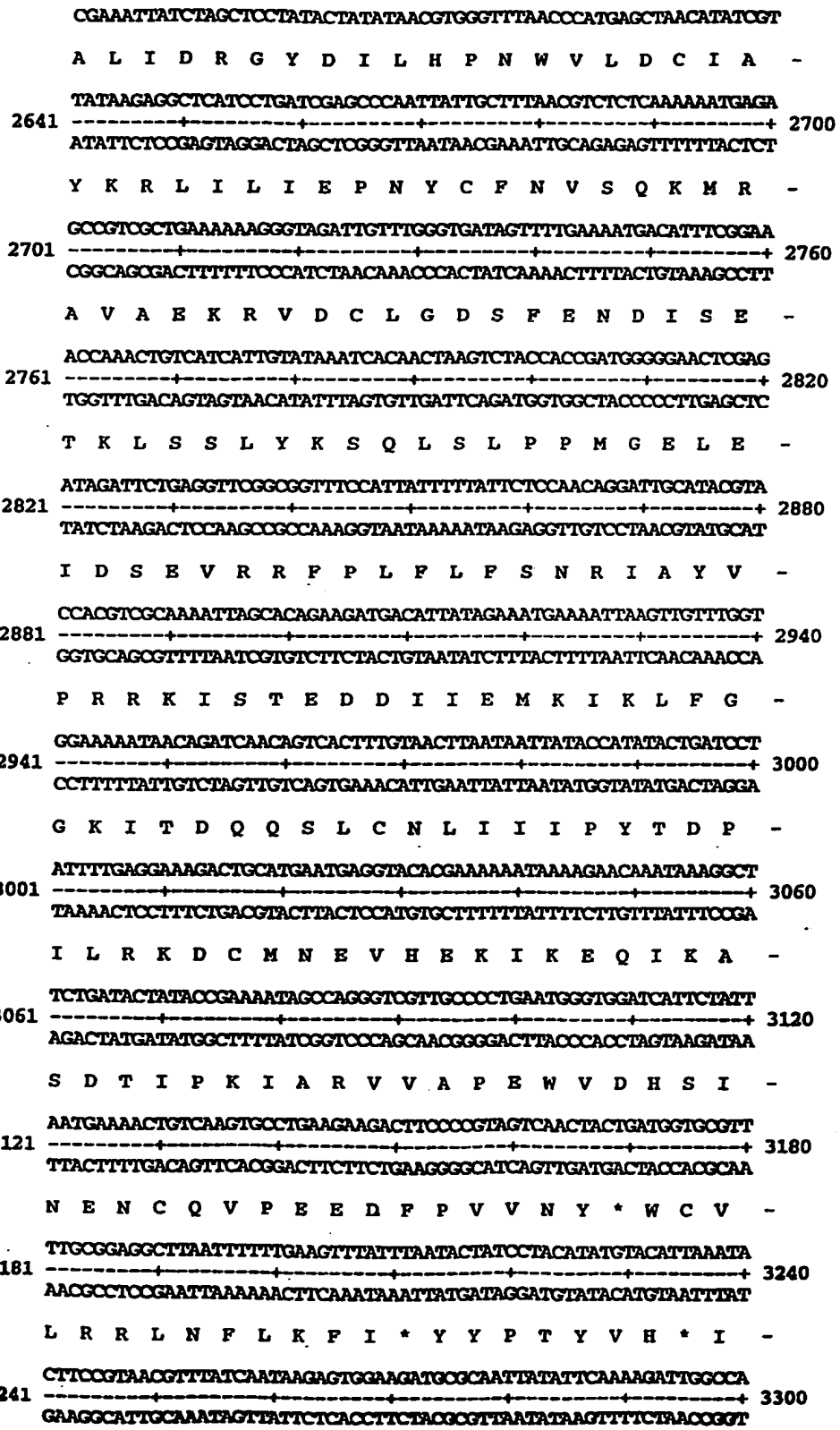

Figure 6 (Continued)

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     CAGTTAATTGAATTCCTTTTTTA

     V  N  *  L  K  E  K  -
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# INTERNATIONAL SEARCH REPORT

Internat' I Application No
PCT/GB 98/00095

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/68 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 94 17189 A (SALK INST FOR BIOLOGICAL STUDI) 4 August 1994<br>see claims 19-22<br>--- | 1-28 |
| X | POLTORATSKY, V.P. ET AL.: "human DNA activated protein kinase (DNA-PK) is homologous to phosphatidylinositol kinase."<br>THE JOURNAL OF IMMUNOLOGY, 1995,<br>pages 4529-44533, XP002065031<br>see the whole document<br>---<br>-/-- | 7 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 May 1998 | 19. 06. 1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Hoekstra, S |

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# INTERNATIONAL SEARCH REPORT

Internat d Application No
PCT/GB 98/00095

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | HIDAKA, H. ET AL.: "Methods in Enzymology volume 201; Protein phosphorylation part B analysis of protein phophorylation, protein kinas inhibitors and protein phosphatases; Ed. T. Hunter and B.M. Sefton; Chapter 27: "Properties and use of H-series compounds as protein kinase inhibitors" 1991 , ACADEMIC PRESS , SAN DIEGO XP002065037 see page 328 - page 339 --- | 7 |
| X | WEI, Y-F, ET AL.: "Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination" MOLECULAR AND CLELLULAR BIOLOGY, vol. 15, no. 6, June 1995, pages 3206-3216, XP002065032 see the whole document --- | 12 |
| Y | GAKEN, JOOP A. ET AL: "Efficient retroviral infection of mammalian cells is blocked by inhibition of poly(ADP-ribose) polymerase activity" J. VIROL. (1996), 70(6), 3992-4000 CODEN: JOVIAM;ISSN: 0022-538X, XP002061469 see the whole document --- | 1-28 |
| Y | KACZMARSKI, WOJCIECH ET AL: "Lupus autoantigen Ku protein binds HIV-1 TAR RNA in vitro" BIOCHEM. BIOPHYS. RES. COMMUN. (1993), 196(2), 935-42 CODEN: BBRCA9;ISSN: 0006-291X, XP002061470 see the whole document --- | 1-28 |
| P,X | GRAWUNDER, U. ET AL.: "Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells" NATURE, vol. 388, 31 July 1997, pages 492-495, XP002065033 see the whole document --- | 1-28 |
| P,X | CRITCHLOW, S. E. ET AL.: "Mammalian double strand break repair protein XRCC4 interacts with DNA ligase IV." CURRENT BIOLOGY, vol. 7, no. 8, 1 August 1997, page 588-598 XP002065034 see the whole document --- -/-- | 1-28 |

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# INTERNATIONAL SEARCH REPORT

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PCT/GB 98/00095

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | TOMKINSON, A.E. ET AL.: "structure and function of mammalian DNA ligases" MUTAT. RES., vol. 407, no. 1, January 1998, pages 1-9, XP002065035 see the whole document | 8-10 |
| A | WO 96 30524 A (HUMAN GENOME SCIENCES INC ;WEI YING FEI (US); YU GUO LIANG (US); H) 3 October 1996 | 1-28 |
| A | WO 95 10288 A (BAYLOR COLLEGE MEDICINE) 20 April 1995 see claim 1 | 1-28 |
| A | SALLES B ET AL: "RAPID QUANTIFICATION OF DNA REPAIR SYNTHESIS IN CELL EXTRACTS" ANALYTICAL BIOCHEMISTRY, vol. 215, 1993, pages 304-306, XP002054958 see the whole document | 1-28 |
| A | HARTLEY, K. ET AL.: "DNA-dependent protein kinase catalytic subunit: A relative of phosphatidylinositol 3-kinase and the Ataxia telangiectasia gene product" CELL, vol. 82, 8 September 1995, pages 849-856, XP002065036 see abstract; figures 1-28 | 1-28 |

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# INTERNATIONAL SEARCH REPORT

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PCT/GB 98/00095

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 20, 23 and 26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the modulating compound.

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Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/GB 98/00095

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9417189 A | 04-08-1994 | CA 2132452 A | 04-08-1994 |
| | | EP 0632832 A | 11-01-1995 |
| | | JP 7505057 T | 08-06-1995 |
| | | US 5627064 A | 06-05-1997 |
| | | US 5686412 A | 11-11-1997 |
| WO 9630524 A | 03-10-1996 | AU 2201695 A | 16-10-1996 |
| WO 9510288 A | 20-04-1995 | AU 7972594 A | 04-05-1995 |